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Rapid remodeling of lipids and transcriptome in the diatom *Phaeodactylum tricornutum* in response to defense related decadienal

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Dedication

To my Dad and Mom, who have always believed in me and kept me strong

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Rapid remodeling of lipids and transcriptome in the diatom *Phaeodactylum tricornutum* in response to defense related decadienal

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Abstract

Diatoms rapidly release extracellular oxylipins (oxygenated lipids) including polyunsaturated aldehydes (PUAs) upon cell damage, as a defense response to inhibit reproduction in many herbivores. PUAs are synthesized from membrane phospholipids. In diatoms, PUAs rapidly increase intracellular Ca^{2+} and nitric oxide (NO) levels and affect gene expression and physiology. Treatment of the marine diatom *Phaeodacylum tricornutum*, with the PUA, decadienal (DD, 10 µM) altered lipid compositions. In cells treated for 3 hr, nine out of ten measured saturated and unsaturated fatty acids declined (range of 0.5-0.7 fold of solvent control levels). At 6 hr, most fatty acid species 14:0, 18:0, 20:5 remained at lower levels while 18:1 and 18:2 increased (1.12 fold and 1.46 fold respectively). Phospholipids in DD treated cells at 3 hr showed decline in PG[1] (0.69 fold) and PS[1] (0.36 fold) whereas PE[1] increased (1.79 fold). At 6 hr, PI[1] (0.79 fold), PS[1] (0.28 fold) and LPG[1] (0.56 fold) declined whereas PC[1] (1.21 fold) and PE[1] (2.7 fold) increased. There were no effects on abundant chloroplast glycolipids (DGDG/MGDG)[1]. Molecular

[1] PG= phosphotidylglycerol; PS= phosphotidylserine; PE= phosphatidylethanolamine; PI= phosphotidylinositol; PS=phosphotidylserine; LPG= lyso phosphotidylglycerol; PC= phosphotidylcholine; DGDG= digalactosyl diacylglycerol; MGDG= monogalactosyl diacylglycerol

species composition of phospholipids showed a greater decline in polyunsaturated fatty acids than monounsaturated fatty acids and saturated fatty acids, which indicates a shift towards saturated fatty acids that may protect membranes from oxidative stress. C-28 brassicasterol declined 0.86 fold at 3hr whereas there was no change at 6hr. Non-polar lipids increased by 1.16 fold and 1.38 fold at 3hr and 6hr. Decreased membrane permeability in DD treated cells was suggested by reduced uptake of cytological dyes. To our knowledge, this is the first report documenting novel rapid (within 6 hr) phospholipid changes in response to PUA suggesting early membrane lipid remodeling to aid in adaptation to PUA stress in algae. Transcriptome analysis at 3hr and 6hr of DD treatment indicated differential regulation of a subset of genes in fatty acid metabolism pathway in co-ordination with genes in multiple metabolic pathways. This suggests that rapid lipid remodeling may be regulated at transcriptome levels to help adapt to decadienal stress.

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Chapter 1: Introduction

Marine phytoplankton are the autotrophic component of the plankton community that form the basis of marine food webs and carry out about 50% of primary productivity on earth (Bowler, 2010). Phytoplankton are composed of photosynthetic prokaryotes and eukaryotes, diatoms being the most abundant and highly diversified group with more than 200,000 species classified as radial or pennate based on their symmetry. They can range in size from micro-meters to millimeters and can exist as single cells, colonies or chains (Bowler et al., 2010). They are the key players involved in biogeochemical cycling of important nutrients such as carbon, nitrogen, iron, phosphorus and silica (Allen et al., 2006). They use silicic acid dissolved in water to construct their cell wall called frustules. Most of the silica entering the ocean has been predicted to be included into diatom cell walls about 40 times before sinking to the seafloor (Treguer et al., 1995). A variety of novel metabolic pathways have been acquired by diatoms during their evolution (Amburst et al., 2004; Fabris et al., 2012; Prihoda et al., 2012). Photosynthesis related pathways in diatoms can provide an evolutionary link between lower photosynthetic organisms and higher plants and a better understanding of these processes. Since diatoms are known to contain genes not only encoding components of photosynthesis but also certain genes typical of animal cells (Amburst et. al 2004, Prihoda et. al, 2012), studying underlying molecular mechanisms will also help link these processes to animals.

Despite their importance, the molecular mechanisms underlying diatom ecological success and their adaptation to very wide range of habitats and environmental conditions are largely unknown. Nonetheless tremendous progress had been made over the last decade with the genome sequencing of a centric diatom *Cyclotella nana* (reported as *Thalassioria pseudonana)* (Amburst et al., 2004) and a pennate diatom *Phaeodactylum tricornutum* (Scala et al., 2002; Bowler et al., 2008) and the generation of a diatom EST database (Maheshwari et al., 2005, 2009, 2010). With the development and availability of various

molecular tools such as expression vectors, genetic transformation and gene silencing tools (Apt et al., 1996; Falciatore et al., 1999; Riso et al., 2009) and identification of reference genes for gene expression studies (Siaut et al., 2007), molecular mechanisms underlying various biological processes can be studied. Since diatoms are of increasing interest due to their biochemical characteristics and application value, advances have been made in various biochemical and analytical methods to isolate and analyze bioactive metabolites such as lipids including PUFAs, carotenoids, phytosterols, vitamins, antioxidants and other natural compounds (Li et al., 2014; Kumari et al., 2013; Gimpel et al., 2015). Further advances in proteomics tools and techniques (Nunn et al., 2009; Rossenwasser et al., 2014) make diatoms a good model to relate the transcriptome and the proteome to dissect various metabolic pathways associated with biochemical and physiological responses to help understand evolutionary and ecological success of diatoms in oceans.

Since diatoms are dominant members of bloom formations, it is important to understand the mechanism and regulation of massive blooms formations and their termination by diatoms due to environmental factors (light, temperature and nutrients) and biotic interaction with grazers, bacteria and viruses (Falkowski et al., 1998; Suttle et al., 2007). This would help inform strategies to prevent harmful algal blooms that can be toxic for aquatic life. Diatoms play a crucial role in fixing atmospheric CO_2, cycling nutrients and are a potential source of commercial products such as polyunsaturated fatty acids and pigments in health, food and biofuels industries (Hu et al., 2008), pointing towards a need for better understanding of fundamental features of their biology.

Stress in diatoms and adaptive strategies

Defense is an important mechanism which helps organisms react to unfavorable conditions and develop resistance and also signal distant cells to prepare themselves for stress environment. Since diatoms are dominant primary producers, it is important to study how these species defend themselves to maintain their population levels. Studies have suggested that diatoms utilize sophisticated mechanisms to monitor and adapt to various

environmental stress conditions (Falciatore et al., 2000; Vardi et al., 2006), regulating the fate of blooms and their success in oceans. Diatoms utilize chemical signals to enhance their defense capacity against grazers and pathogens (Wolfe et al., 1997) and outcompete other phytoplankton species (Keating et al., 1977; Leflaive et al., 2009).

Grazing or mechanical cell disruption in diatoms induced rapid production of diatom derived unsaturated aldehydes that inhibit the reproduction of potential grazers/copepods (Miralto et al., 1999; Ribalet et al., 2007) and also control their own population fate. Several polyunsaturated aldehydes (PUAs) and polyunsaturated fatty acids (PUFAs), broader category known as oxylipins (oxygenated lipids) are produced by diatoms upon cell damage/grazing. These compounds play a complex role in intra and interspecific interactions, and in grazer defense processes (Leflaive et al., 2009). They also act as infochemicals that induce resistance in diatoms at sublethal levels or can induce cell death at higher doses (Figure1.1). The most studied PUA is Decadienal (DD), known to elicit various responses in grazers and in diatoms themselves.

Known effects of decadienal in grazers

Miralto et al (1999) reported that the hatching success of copepods was compromised in copepods that fed on blooms dominated by diatoms. The rate of hatching eggs was 12% compared to the post bloom conditions when there was 90% hatching success. The authors reported structures of three poly unsaturated aldehydes (PUAs) namely *trans*-4-*cis*-7-*cis*-decatrienal, 2-*trans*-4-*trans*-7-*cis*-decatrienal and 2-*trans*-4-*trans*-decadienal (DD)**,** which were isolated from diatoms *Thalossiosira rotula*, *Skeletonema costatum* and *Pseudo-nitzschia delicatissima*. These compounds were shown to be responsible for arresting embryonic development in copepods and sea urchins and had anti-proliferative and apoptotic effects on human carcinoma cells. Studies showed that copepods may respond differently to same diatom species. Ianora (2005) reported the copepods *Acartia clausi*, *Centropages typicus*, *Calanus helgolandicus* and *Temora*

stylifera had different reproductive responses to the diatom *Thalossiosira rotula*. While responses were extreme in *Centropages typicus*, in *Temora stylifera* there was reduced effect whereas there was no effect in *Acartia clausi and Calanus helgolandicus*. Poulet et al. (2007a) could not relate reproductive failure to PUAs and other polyunsaturated fatty acids (PUFAs) in his study on effect of 20 different algal diets on egg production and hatching success in *Calanus helgolandicus.* Taylor et al. (2007) examined the effect of diatom derived oxylipins and that of oxylipin containing diatom diets in the reproductive success of the copepod *Tisbe holothuriae* showed that among all PUAs examined, 9.3 µM decadienal was most toxic and had a greatest effect on *Tisbe holothuriae* nauplii followed by adult males and then adult females. They also showed that there was no negative impact of short term exposure of PUA producing diatom (*Skeletonema marinoi* and *Melosira nummuloides*) diets on survival and development of naupliar stages to adulthood. Ka et al. (2014) showed that decadienal directly affected survivorship and reproductive success of the calanoid copepod *Temora stylifera*. DD induced mortality in both males and females with higher mortality in males at concentrations above 3 $\mu g\ mL^{-1}$. Egg production and hatching rates decreased with increasing concentration of DD. Their study also showed that presence of PUAs from diatoms may increase feeding rates in copepods.

In summary, although a number of studies have reported negative impacts of oxylipins including PUAs and that of oxylipin containing diatom diets on various grazers, there has been some reports that didn't see these effects (reviewed by Ianora et al., 2010). This suggested that these effects can be very much dependent on species of grazers as well as species of diatoms along with various interacting environmental factors or variations in the setup of laboratory experiments.

Romano et al. (2011) showed that nitric oxide (NO) regulates expression of stress and apoptosis genes in the embryos of sea urchin *Paracentrotus lividus* in response to decadienal in a dose dependent manner. At low concentrations of decadienal, NO induces expression of *hsp70* gene thereby protecting embryos against toxic effects of aldehyde whereas at higher levels of decadienal, apoptosis was initiated with increased expression

of *caspase-8* gene. Varrella et al. (2014, 2016) studied dose dependent effects of PUAs (decadienal, octadienal, heptadienal) on gene expression networks in *Paracentrotus lividus* showed that PUAs affect genes in many functional processes such as stress, differentiation, development, skeletogenesis and detoxification. Carotenuto et al. (2013) showed that the copepod *C. helgolandicus* when fed on toxic diatom diet (PUA producing *S marinoi*), genes in biological processes such as response to stimuli, signal transduction and protein folding were affected, mediating the physiological response. These studies showed that integrated network of genes at transcript levels regulate physiological responses of copepods to PUAs. Recent studies by Wolfram et al. (2014) to understand mechanism of PUA uptake and accumulation in adult females of *Acartia tonsa* using a PUA derived fluorescent probe showed that PUA selectively accumulates in gonads of the copepod suggesting interaction of PUAs with copepod reproductive tissue causing teratogenic effects.

Known effects of decadienal in diatoms

Many diatoms are known to produce PUAs or other oxygenated lipids upon wounding or mechanical cell damage (Wichard et al., 2005, 2007). PUAs have been studied for their role as infochemicals mediating cell-cell communication in diatoms. Casotti et al. (2005) proposed that PUAs apart from being defensive molecules against grazers can also act as active internal controls during unfavorable conditions determining biomass, structure and dynamics of phytoplankton communities. Ribalet et al. (2007a, 2007b) estimated that PUAs released from diatoms could be in the range of 0.5µm to 46µm at a distance of 1µm-100 µm from the cell surface and could very well affect growth and performance of the surrounding organisms. They found that amount of PUA production was much higher in stationary phase compared to exponential culture and especially enhanced in nutrient limited (N and P limited) *Skeletonema marinoi* cultures.

Decadienal (DD) is the most studied among various other PUAs released by diatoms upon cell damage. Certain diatoms, such as *Phaeodactylum tricornutum* release non-volatile oxylipins such as 9-ONDE and 12-ODTE that are known to have similar effects of inhibition of grazer reproduction (Pohnert, 2002). The mechanism of cellular reaction to DD has been studied in detail in the diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* (Figure 1.2). DD triggered dose dependent increase in intracellular Ca^{+2} that led to production of Nitric oxide (NO) by calcium dependent nitric oxide synthase like activity (Vardi et al., 2006, 2008). Fate of DD exposed cells were dose dependent: sublethal doses (660nM-13.2µM) of DD triggered signaling phenomenon leading to induced resistance whereas higher doses (>20µM) induced programmed cell death. Cells receiving sublethal doses of DD were further resistant to lethal doses of DD showing increased growth and photosynthetic efficiency (Vardi et al., 2006). Studies also indicated that DD induced stress could be transmitted to non-exposed cells by a diffusible signal from stressed cells (Leflaive et al., 2009, Vardi et. al, 2006) that led to NO production in the nearby population. Hence monitoring infochemical concentrations provides a refined mechanism wherein subthreshold levels could serve as early warning protective signal to cells nearby whereas lethal doses would initiate the cascade for cell death and bloom termination helping in saving aquatic life from toxic effects of harmful algal blooms.

Vardi et al. (2008) followed a functional genomics approach to dissect the signaling pathway involved in sensing of PUA to regulate diatom cell fate and identified a DD induced gene PtNOA (nitric oxide associated protein) in *P. tricornutum*. This protein belongs to highly conserved YqeH subfamily of GTP binding proteins and thought to play a role in ribosome biogenesis (Uicker et al., 2007), sporulation and nitric oxide generation (Guo et al., 2003). Overexpression of PtNOA gene led to higher NO production, reduced growth and impaired photosynthetic efficiency compared to wild type cells. PtNOA transformants were hypersensitive to sublethal levels of the aldehyde. Altered expression

of superoxide dismutase, metacaspases and caspases, which are key components of stress and cell death pathways were observed. These transformants were also compromised in their ability to form a biofilm by a reduced attachment strength compared to wild type cells (Vardi et al., 2008). These changes may confer an advantage to the cells to be able to float away from site or area of stress/ unfavorable conditions due to reduced adhesion to the substrate.

Since production of PUAs and polyunsaturated fatty acids (PUFAs) by these microorganisms for their defense is dependent on the availability of precursor lipids, it is important to understand how various abiotic and biotic factors can affect their lipid content and composition. Various studies have documented the effect of abiotic factors (light, temperature, nutrient, heavy metal, etc.) on lipids and fatty acid composition in unicellular algae (Sharma et al., 2012). However, in regard to biotic stress, studies have been limited to reporting rapid fatty acids changes in response to mechanical damage (sonication) in number of PUA producing diatoms (Wichard et al., 2007). The present work explored a novel area of studying changes in lipids in distant undamaged (healthy) cells in response to DD. Vardi et al. (2007) showed a possible relay of signal from DD exposed NO producing cells to distant unexposed cells, which respond by producing NO. Hence we wanted to understand how lipid changes in response to DD can help nearby populations adapt to unfavorable conditions that may help develop resistance or induce other defense responses. Since overall lipid content in diatoms determines food quality for grazers, this study would help enhance knowledge on factors determining phytoplankton community structure during and after algal blooms. Algal biomass has been largely utilized for various commercial products and one of the challenges in growing outdoor cultures for scaling up the biomass is crashing of cultures due to grazers or other environmental factors. We suggest that our study would also provide information on cost effective utilization of the algal biomass.

Effect of abiotic stress on changes in lipids in algae

Sharma et al. (2012) summarized the effects of various abiotic stresses such as nutrient starvation, temperature, light, pH, heavy metals, salt and UV on lipid production and FA composition in microalgae. With most kinds of stress there is a decline in cell division and reduced algal growth not requiring any new synthesis of membrane compounds. Cells then divert and deposit fatty acid in form of storage lipids (TAGs). Overall various studies have reported that with nutrient limitation (starvation) there is an increase in lipid production in microalgae. *Stephanodiscus minutulus* when grown under nitrogen, silicon and phosphorous limitation led to increase in storage lipids (TAGs) and decrease in polar lipids assayed each day for 11 days (Lynn et al., 2000). In *Chlamydomonas moewusii* nutrient limitation led to decreased PUFAs (16:3, 16:4 and 18:3) whereas overall levels of 16:1 and 18:1 FA increased (Arisz et al., 2000) measured between 1-5 weeks of growth. Several studies have reported accumulation of lipids (mostly neutral lipids) in response to nitrogen deficiency in various strains of microalgae (Yeh et al., 2011; Praveen Kumar et al., 2012; Hsieh et al., 2009). There was a significant rise in lipid production upon nitrogen limitation in several green algae and diatoms including *P. tricornutum* (Hu et al., 2006; Yang et al., 2013; Alipanah et al., 2015). Higher levels of neutral lipids and higher proportions of saturated and mono unsaturated FAs were produced in *Cyclotella cryptica* due to silicon deficiency within 4-12 hours (Roessler, 1988). There was an increase in TAGs (from 69 to75%) and phospholipids (from 6 to 8%) in response to reduced nitrogen concentrations in *P. tricornutum* within 24 hr-7 days (Alonso et al., 2000). Phosphorus limitation led to increased lipid content mainly TAGs in various species such as *P. tricornutum*, *Chaetoceros* sp, *Isocrysis galbana* and *Pavlola lutheri* but decreased content in *Nannochloris atomus* and *Tetraselmis* sp within 24-48 hours. There was an increase in production of 16:0 and 18:1 and decrease in 18:4, 20:5 and 22:6 levels (Reitan et.al, 1994, Yang et. al, 2014).

Temperature stress had a major influence on fatty acid composition of microalgae with the unsaturated fatty acids increasing with decreasing temperature and saturated fatty acids increasing with increasing temperature to maintain biological functioning of processes of the cell (Renaud et al., 2002; Lynch et al., 1982; Murata et al., 1975; Sato et al., 1980). In *P. tricornutum,* a temperature shift from 25 deg C to 10 deg C for 12 hours led to highest yields of polyunsaturated fatty acids (PUFAs) and eicosapentanoic acid (EPA) per unit dry mass, both being raised to 120% above the control (Jiang et. al, 2004). It was concluded that significant increase in PUFAs and EPA yields can be achieved by lowering culture temperature in microalgae.

Dunaliella spp. have been studied for the effect of salinity on lipid composition as they have ability to tolerate and grow at varied salt conditions (Azachi et al., 2002; Takagi et al., 2006; Xu et al., 1997). A significantly higher ratio of C18 (mostly unsaturated) to C16 (mostly saturated) occurred at high salt concentrations. Total saturated and monounsaturated fatty acid increased with increased salt concentrations whereas the proportion of PUFAs decreased. In *Nitzchia laevis* which is known to produce high amounts of EPA, degree of FA unsaturation of both neutral and polar lipids increased sharply under moderately high salt concentrations (10 to 20g/L) but decreased at much higher levels of salt (30g/L) as measured during early stationary phase of growth (Chen et al., 2008).

Effects of pH and heavy metals on lipid composition have also been studied in microalgae. Increased TAG accumulation and decreased membrane lipids in *Chlorella* (Guckert et al., 1990) was reported with increasing pH. Alkaline pH inhibits growth of microalgae thus diverting the energy to form TAG. A study which compared FA composition between an acidic *Chlamydomonas* sp. (that isolated from acidic lake) and *C. reinhardtii* showed that the acidic species had more saturated polar lipids and higher proportion of TAGs. The increased saturation of membrane fatty acids in acidic species

was suggested to be an adaptation at low pH to decrease membrane lipid fluidity (Tatsuzawa et al., 1996). Increased lipid content has been observed in certain microalgae due to heavy metal stress such as cadmium, iron and copper. Cadmium was shown to increase total lipid content in *Euglena gracilis*. There was no change in total phospholipid content however there was an increase in phosphotidylglycerol (PG) (Tatsuzawa et al., 1996). Another effect of iron was seen on lipid accumulation in *Chlorella vulgaris* where total lipid content increased due to iron stress (Liu et al., 2008).

Light regulates growth of microalgae and different intensity and wavelength are required for different microalgae that alters lipid composition in these algae by altering their lipid metabolism. It was shown that high light led to oxidative damage of PUFA and altered level of C16: 1 fatty acid in various microalgae (Harwood, 1998). There was an increase in neutral lipids mainly TAGs and total phospholipid content decreased as reported in green alga *Cladophora* (Napolitano, 1994). On other hand low light was seen to induce formation of membrane polar lipids (Brown et al., 1996; Khotimchenko et al., 2005; Napolitano, 1994; Orcutt et al., 1974). In red algae *Tichocarpus crinitus* low light intensity led to increased levels of certain membrane lipids whereas high light increased levels of TAG measured after 21 days (Khotimchenko et. al, 2005). TAG production in high light intensity supposedly protects the cells by increased FA synthesis (stored as TAGs) to regenerate electron acceptor pool. Light/dark cycles at different growth phases also effect lipid composition. In a study on a diatom *Thalossiosira pseudonana* it was seen that higher amounts of TAGs with saturated and monounsaturated fatty acids accumulated in cultures grown to stationary phase under continuous light or 12:12 strong light/dark cycle compared to low light (Brown et. al, 1996). However at exponential growth phase, high light showed increased PUFA content indicating the role of growth phase on lipid accumulation in certain microalgae species.

Effect of UV radiation has also seen to change lipid composition and accumulation in various microalgae species. *P. tricornutum* and *C. mulleri* were studied for the effect of UV radiation on their fatty acid composition. With UV-A treatment in *P. tricornutum* EPA and PUFA increased while monounsaturated FA decreased whereas in *C. mulleri* percentage of monounsaturated FA increased and EPA and PUFA decreased under the combined effect of UV-A and UV-B treatment (Liang et. al, 2006).

Hence it is clear that the effect of various abiotic stresses on lipid composition in different microalgae species varies greatly and they react by either producing different fatty acids or changing the composition of fatty acids to adapt to these stresses. Most of the abiotic stress studies were long term, i.e. stresses were applied for 48-72 hours and sometimes for 7-14 days before lipids were evaluated. Although a number of studies have been documented changes in lipids in microalgae with various abiotic stresses, limited light has been shed on the effect of biotic stress such as wounding or grazing by predators on changes in lipids in microalgae within a short time scale (6-12 hours).

Effect of mechanical wounding stress on lipids in algae

Many freshwater and marine studies have documented the impact of phytoplankton lipids and free fatty acids on predator success. Polyunsaturated fatty acids along with steroids have been identified as key factors that influence growth, survival and reproduction of predators. Copepods retain polyunsaturated fatty acids (PUFAs) such as EPA and DHA at all stages of development (Wichard et al., 2007). The composition and amount of lipids in phytoplankton have a major effect on the health and reproductive success of zooplanktons (Pohnert 2005; Vehmaa et al., 2011; Miralto et al., 1999). Diatoms are an important source of nutrition since they produce large quantities of PUFAs like EPA and oils (Martins et al., 2013). Overall, diatoms have been under-represented in studies characterizing fatty acids in microorganisms. Limited studies have been done to understand

how biotic stress like cell damage/mechanical wounding affect lipids and fatty acid composition in these photosynthetic microorganisms especially in a short time scale.

Studies show that some diatoms upon mechanical wounding react with rapid release of polyunsaturated aldehydes (PUAs) (Wichard et al., 2007). This defense response activated during predator feeding has been shown to decrease predator hatching success and offspring survival. These PUAs are derived from polyunsaturated fatty acids (PUFAs) that are released from phospholipids and galactolipids (PLs and GLs) after cell disruption. Due to fatty acid degradation upon cell breakage it is possible that diatom diet lacks essential fatty acids and copepods can no longer benefit from these PUFAs (Wichard et al., 2007).

Wichard et al. (2007) has shown that in an isolate of *Thalassiosira rotula* that produces PUA upon mechanical wounding, the amount of eicosapentanoic acid (EPA) rapidly depletes to 30% of its initial content within 15-30 minutes. Similar depletion was seen in PUFAs C16:3 and C16:4. These PUFAs are known to be precursors of certain PUAs. However saturated fatty acids remain unaffected. In another diatom *Thalassiosira pseudonana* that doesn't produce PUA upon wounding, no changes in PUFAs were observed after wounding. This was confirmed with several PUA producing and non-producing diatoms where rapid depletion of PUFAs was seen with cell disruption after 30 minutes. *Phaeodactylum tricornutum* is known to be a non-PUA producer (Wichard et al., 2005, Lefliave and Ten-Hage, 2009) similar to *Thalassiosira pseudonana* but lipid changes in this diatom in response to cell damage has not been reported yet.

Majority of polyunsaturated fatty acids as studied in some diatoms are stored in polar lipids such as PLs and GLs while small amount are detected in non-polar lipid fractions. Those polyunsaturated fatty acids that are stored in triglycerides are not released and transformed and could account for remaining PUFAs after wounding (Wichard et al., 2007). Further

experiments done by Wichard et al. (2007) on fatty acid supplementation showed that when algal cells were supplemented externally with EPA (C20:5), heptadienal and decatrienal production increased significantly upon wounding. Also external addition of arachidonic acid (C20:4, ARA) increased decadienal production. This shows that PUA production is limited by availability of fatty acids. Further experiments relating to effect of EPA depletion on hatching success of a copepod when fed with a PUA producer diatom and a non-PUA producer diatom showed that EPA supplementation of PUA producing diatom diet benefited hatching success of copepod whereas remain unaffected in case of non PUA producing diatom diet.

Cell damage in *Thalassiosira rotula* results in release of free mono and polyunsaturated fatty acids by the action of phospholipases. Eicosatetraenoic and Eicosapentaenoic acid are further converted by action of lipoxygenases to reactive aldehydes 2, 4 decadienal and 2, 4, 7 decatrienal (Pohnert, 2002) (Figure 1.3). It was reported that mainly saturated free fatty acids are present in intact diatom *T. rotula* whereas amount of free polyunsaturated eicosanoids is drastically increased in first 5 mins after wounding. Free fatty acids extracted 10 minutes after cell disruption through sonication showed an overall strong increase in amount of PUFAs, most profound for 20:5 (EPA) and also levels of unsaturated C16 and C18 fatty acids increased. Other studies done on diatoms *Skeletonema costatum* and *Pseudo-nitzchia* sp. also showed that no free fatty acids were present in the intact cells whereas increased free fatty acids in wounded cells. Hence diatoms rely on phospholipids and transformation of C20 fatty acids to form PUAs as an activated defense.

Some marine algae (diatoms) may not produce PUAs but other bioactive compounds from precursor fatty acids (collectively called as oxylipins) which have anti-proliferative properties similar to PUAs and can also help in defense. These compounds have a characteristic α, β, γ, δ-unsaturated aldehyde group and are potent Michael

acceptors. These anti-proliferative compounds were studied to be main chemical factors involved in diatom defense against grazers/copepods (Miralto et al., 1999; Pohnert et al., 2002). Structures of various oxylipins are shown in Figure 1.4. Production of various volatile and non-volatile oxylipins is very much species and strain specific and also depends on various environmental factors (Pohnert 2005 and Pohnert et al., 2002).

Diatoms *Gomphonema parvulum, Asterionella formosa* and *Phaeodactylum tricornutum* were reported to produce oxygenated products such as 9-oxo-(5Z, 7E)-nonadienoic acid (9-ONDE) and 12-oxo-(5Z, 8Z, 10E)-dodecatrienoic acid (12-ODTE) from precursor EPA upon wounding (Figure 1.5). These reactive molecules similar to PUAs have reactive α, β, γ, δ-unsaturated aldehyde group and were shown to have anti-proliferative properties similar to PUAs.

***Phaeodactylum tricornutum*: Choice of organism under study for this project**

Phaeodactylum tricornutum is a heterkont marine diatom belonging to class Bacillariophyceae. It can exist in different morphotypes such as fusiform, oval, triradiate and a rare form called cruciform and environmental conditions can stimulate changes in their form from one to another (Martino et al., 2007; He et al., 2014). *Phaeodactylum* can grow in the absence of silica and survive without silicified cell walls unlike most diatoms and it ranges from Germany and France in Europe to Nova Scotia in North America. *Phaeodactylum tricornutum* whose genome has been sequenced and molecular toolbox (expression vectors, transformation and gene silencing tools) was developed makes it a good model for molecular genetics and physiological and biochemical studies (Siaut et al., 2007; Apt et al., 1996; Riso et al., 2009).

Phaeodactylum tricornutum is relatively easy to culture and maintain under laboratory conditions, grows fast (doubling time ~1.5 days), easy to harvest (centrifuge/collection on filter) and shows cellular reactions to the model (most studied)

PUA, Decadienal. *P. tricornutum* is also a good candidate for biofuels and other commercial products with lipid content around 20-30% dry weight under normal conditions (Chisti et al., 2007). It has high content of PUFAs especially eicosapentaenoic acid (EPA) which makes it useful at industrial level for aquaculture feed (Atalah et al., 2007; Hamilton et al., 2014; Martins et al., 2013). A recent study showed potential for bioactive metabolites (pheromones and antimicrobial substances) in local *Phaeodactylum* strain (BMB) isolated from Western Norwegian waters (Prestegard et al., 2016).

Lipid composition and known lipid biosynthesis pathways in P. tricornutum

Domergue et.al (2003) studied fatty acid composition of *P. tricornutum* (UTEX 646) and reported EPA (20:5) to be one of the major fatty acid accounting up to 30%. Other abundant fatty acids were palmitoleic acid ($16:1^{\Delta 9}$; 26%), palmitic acid (16:0; 17%), hexadecatrienoic acid ($16:3^{\Delta 6, 9, 12}$; 10%), and myristic acid (14:0; 5%). Fatty acids such as 16:2, 18:3 and 20:4 were in minority (<1 %) (Figure 1.6).

The pathway for fatty acid synthesis and genes involved has been characterized in *P. tricornutum* (Domergue et al., 2002, 2003). Palmitic and oleic acid are main products of de novo synthesis and EPA is synthesized by desaturation and elongation of oleic acid. Δ5 and the Δ6-desaturases involved in EPA biosynthesis in *P. tricornutum* were characterized and possible biosynthesis routes were proposed (Domergue et al., 2002) (Figure 1.7). EPA can be synthesized by classical omega-6 pathway, omega-3 pathway or a pathway relying on intermediates of both pathways. These desaturases are microsomal enzymes indicating that several steps of EPA biosynthesis take place in endoplasmic reticulum (ER). The most active route likely involved successively delta6-desaturation (18:2 to 18:3), omega3-desaturation (18:3 to 18:4), delta6-elongation (18:4 to 20:4) and a final delta 5-desaturation (20:4 to 20:5) (Figure 1.7).

Synthesis of fatty acid is started by acetyl-CoA in the chloroplast. Conversion of pyruvate, citrate or acetate in plastid and cytosol leads to formation of acetyl-CoA. Acetyl-CoA carboxylase (ACCase) converts acetyl-CoA to malonyl-CoA. In *P. tricornutum* there are two ACCases, one in the plastid (ACC1) and other in the cytosol (ACC2) (Huerlimann et al., 2013). ACC1 converts malonyl-CoA for de novo FA synthesis in the plastid; ACC2 generates malonyl-CoA pool for FA elongation in the cytosol (Nikolau et al., 2003). Malonyl-CoA is condensed and reacts with acetyl-CoA to form acyl-ACP (four carbons) and acyl-ACP further reacts with malonyl-ACP extending two carbons at a time and repeating the four-step reaction cycle (Ryall et al., 2003). Enzymes responsible for these reactions are called fatty acid synthases (FAS). *P. tricornutum* possesses type II fatty acid synthase which is thought to be involved in production of saturated 14:0, 16:0 and 18:0 fatty acids (Domergue et al., 2003). Desaturase enzymes converts saturated to unsaturated fatty acids in presence of oxygen. In *P. tricornutum*, various desaturases have been identified and characterized (Figure 1.7, 1.8).

These fatty acids produced by ACC and FAS can be directly used to produce structural lipids in the chloroplast or are exported out in cytosol for their use in membrane lipids or TAG production. The major components of chloroplast/photosynthetic membranes are glycosylglycerides (glycolipids) such as Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulphoquinovosyldiacylglycerol (SQDG). One of the other special components of the photosynthetic membrane is phosphatidylglycerol (PG). These chloroplast lipids are more abundant than phosphoglycerides. Fatty acid composition in these lipids can vary greatly for different genus and species of diatoms. *P. tricornutum* and *Thalassiosira weissflogii* contain mainly C20/C16 and C18/C16 forms of MGDG and DGDG (75). Plasma membrane and other membrane (ER) phospholipids such as phosphotidyl choline (PC), phosphotidyl ethanolamine (PE), lyso phosphotidyl choline (LPC) and lyso phosphotidyl ethanolamine (LPE) are synthesized by membrane bound desaturases and enzymes in Kennedy pathway

using fatty acid acetyl Co-A pools in the cytosol (Guschina and Harwood 2006; Muhlroth et al., 2013). As shown in Figure 1.6 and 1.7, *P. tricornutum* is abundant in EPA (20:5) and has diverse pathways for its synthesis. Owing to EPA's abundance and importance in *P tricornutum*, its incorporation into lipid classes after its synthesis in the membrane is discussed below. Other free fatty acids depending on their availability and form in which they are present in the cytosol (acyl-CoA pools), could also be incorporated into lipid classes in a similar manner. Free EPA could be linked to PE, PC or diacylglyceryltrimethylhomoserine (DGTS) and then either processed through Kennedy pathway or imported to plastid and incorporated into glycolipids (Guschina et al., 2006; Joyard et al., 2010).

In the Kennedy pathway GPAT results in formation of lysophosphatidic acid (LPA) which is then converted to phosphatidic acid (PA) by lysophosphatidate acyltransferase (LPAAT) (Joyard et al., 2010; Gibellini et al., 2010). Further by the action of phosphatidic acid phosphatase (PAP), Diacylglycerol (DAG) is formed. These PA and DAG along with other phospholipids can act as precursors for the production of sulfolipids and galactosylglycerides. Sulfoquinovosyldiacylglycerol synthase (SQD2), MGD and DGD synthases can process incorporation of EPA into SQDG, MGDG and DGDG. Action of diacylglycerol acyltransferases on DAGs can result in production of TAGs (Figure 1.9).

In *P. tricornutum*, EPA pool can also accumulate as TAGs which mostly occurs during stress or sub optimal conditions. Main contributors of EPA in TAGs are FAs during remodeling of membranes and only small amounts of synthesized EPA is assumed to be incorporated in TAGs. Enzymes such as phospholipase and phospholipid/diacylglycerol acyltransferase (PDAT) can act to convert membrane lipids to TAGs. It was shown that in *P. tricornutum* under nitrogen starvation, remodeling of lipid classes such as PC, PE or galactosylipids contributed to lipid accumulation due to increased PDAT expression (Mus et al., 2013).

Although characterization of fatty acids, lipids classes and their synthesis pathways has been studied to an extent, effect of biotic stress such as cell damage or PUAs such as decadienal on lipids in algae is still very limited.

Regulation of various stresses at transcriptome levels in P. tricornutum

With recent advances in high throughout sequencing and availability of sequenced genome of *P. tricornutum* (Bowler et al., 2008), number of studies have been done to understand transcriptome regulation of various biochemical and physiological changes. Maheshwari et al. (2010) generated cDNA libraries from *P. tricornutum* cells grown under 16 different abiotic stresses/ conditions (nutrients, temperature, salinity) including DD treatments (0.5 and 5 µg/ml for 6 hr). They performed various comparative and functional studies and identified transcripts differentially expressed in response to specific treatments which provided insights into expression pattern of genes involved in various metabolic and regulatory pathways. Recent studies done by Yang et al. (2013, 2014) on nitrogen and phosphorous limitation in *P. tricornutum* showed that various genes involved in ion transport, photosynthesis, carbon cycle, TCA cycle, glycolysis and stress were regulated at transcriptome level to help adapt to nitrogen and phosphorous stress which aids in various biochemical changes leading to neutral lipid accumulation.

Alipanah et al. (2015) also showed that nitrogen stress in *P. tricornutum* is regulated at mRNA levels of genes in multiple metabolic pathways leading to various physiological responses of decreased photosynthesis and chlorophyll content and increased non polar lipids (TAGs). Chauton et al. (2013) showed that genes in TCA and fatty acid biosynthesis cycle are regulated at transcriptome levels over light/dark cycle leading to different fatty acid compositions and levels during light and dark. However most of these studies were over a period of 48-72 hours, studies done by Matthijs et al. (2016) reported early (<20 hours) transcriptome regulation of nitrogen stress response in *P. tricornutum*. Couple of

studies in another organism *Ectocarpus siliculosus* done by Ritter et al. (2014) and Dittami et al. (2009) have revealed extensive reprogramming of transcriptome in response to acclimation to short term (4-8 hr) stresses of copper, NaCl and H_2O_2.

Study done by Wolfram et al. (2015) to identify protein targets of decadienal in *P. tricornutum* revealed that several proteins in photosynthesis, ATP generation and calvin cycle such as fucoxanthin chlorophyll *a*/*c* proteins, ATP synthases, a ribulose-phosphate-3-epimerase (RPE) and a phosphoribulokinase (PRK) were targeted thus affecting its function. This suggested that DD induced physiological changes in diatoms are regulated at proteome level. Rossenwasser et al. (2014) identified approximately 300 redox sensitive proteins mediating response of nitrogen stress induced oxidative stress in *P. tricornutum* indicating that transcriptome and proteome work in cohesion to determine cell fate in response to environmental stress

There were enough convincing reports stating that regulation of transcriptome is involved in adaptation of diatoms to various stresses. Hence after evaluating changes in lipids in response to decadienal over a short time scale of 6 hr (Chapter 2), we wanted understand and correlate lipid changes to transcriptome regulation of genes in various pathways, focusing on lipid metabolism genes (Chapter 3).

Broad aims of this project

Aim1: *S*tudy changes in lipids (fatty acids and lipid classes) in *P. tricornutum* (*Pt* CCMP 2561) in response to decadienal (DD).

Aim2: RNA sequencing to understand transcriptome regulation of genes involved in lipid biosynthesis and metabolism pathways under the effect of decadienal (DD).

Hypothesis

Sublethal dose of DD induces rapid changes in membrane lipids in *P. tricornutum.* These changes may be due to production of downstream oxylipin(s) to signal neighboring cells and/or an adaptation to modify membranes to cope with the DD effect. Further, non-polar lipids may increase to provide a reservoir of long chain fatty acids in rebuilding membranes necessary for survival. These changes may be regulated at transcriptome levels**.**

Significance of this study

- Since effects of PUAs on lipid changes are currently unknown, this novel study can help determine these changes in a short time scale (within 6 hr) and correlate it to changes in the transcriptome.

- Our findings may indicate adaptive remodeling of lipids and other metabolism changes for survival of the algae in response of PUA induced stress conditions.

- This study may give some insights on understanding phytoplankton community structure; determine nutritional transfer efficiency between producers and consumers.

- This study will help in better utilization of algal biomass for cost effective commercial products.

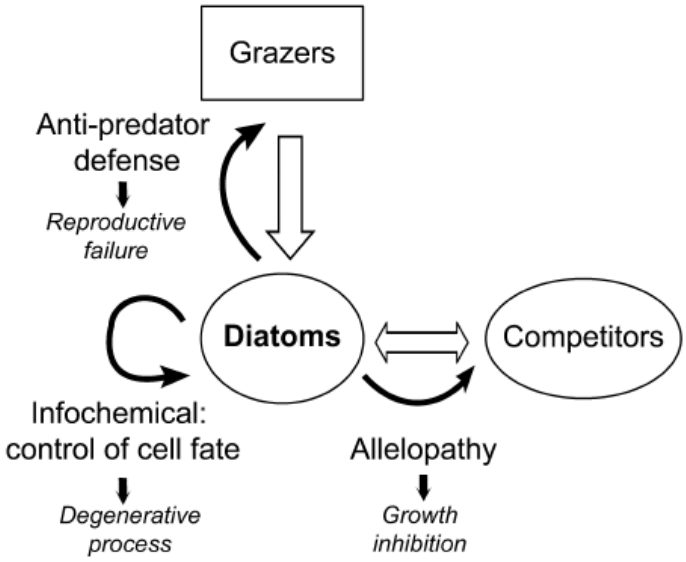


Figure 1.1: Interactions between diatoms and grazers or competitors (indicated by white arrows) that are mediated by production of PUAs such as 2E 4E-decadienal (DD) (indicated by black arrows) (Adapted from Leflaive et al., 2009).

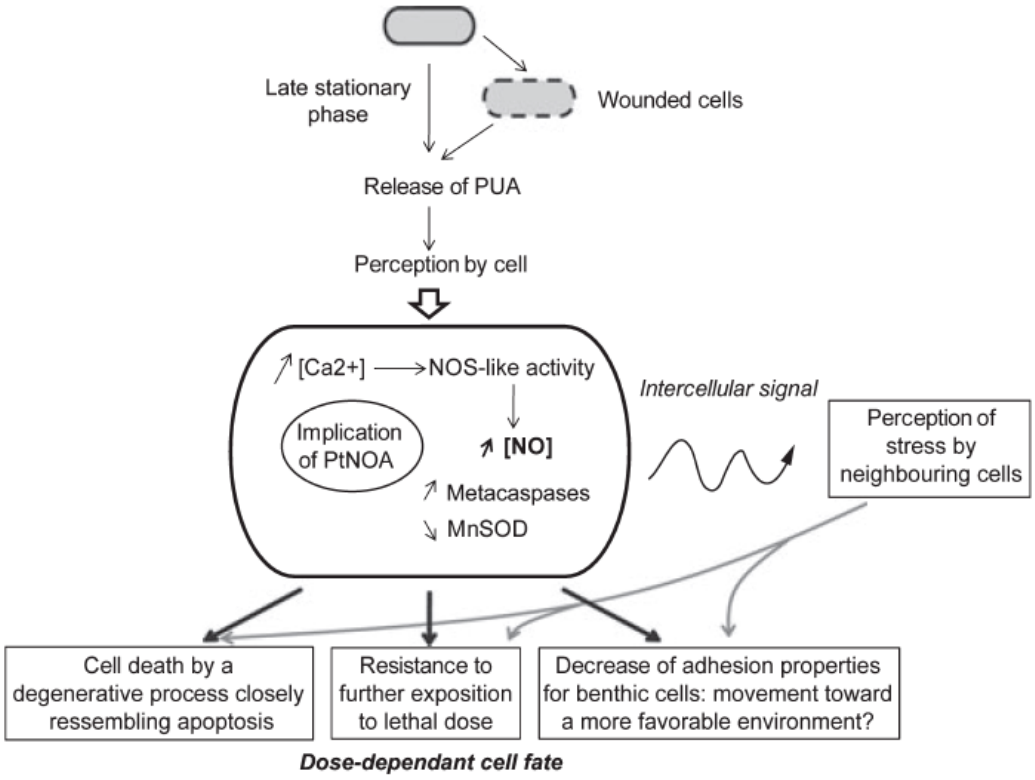


Figure 1.2: Cellular reactions and consequences resulting from the exposure of diatom cell to the polyunsaturated aldehyde decadienal (Adapted from Leflaive et al., 2009).

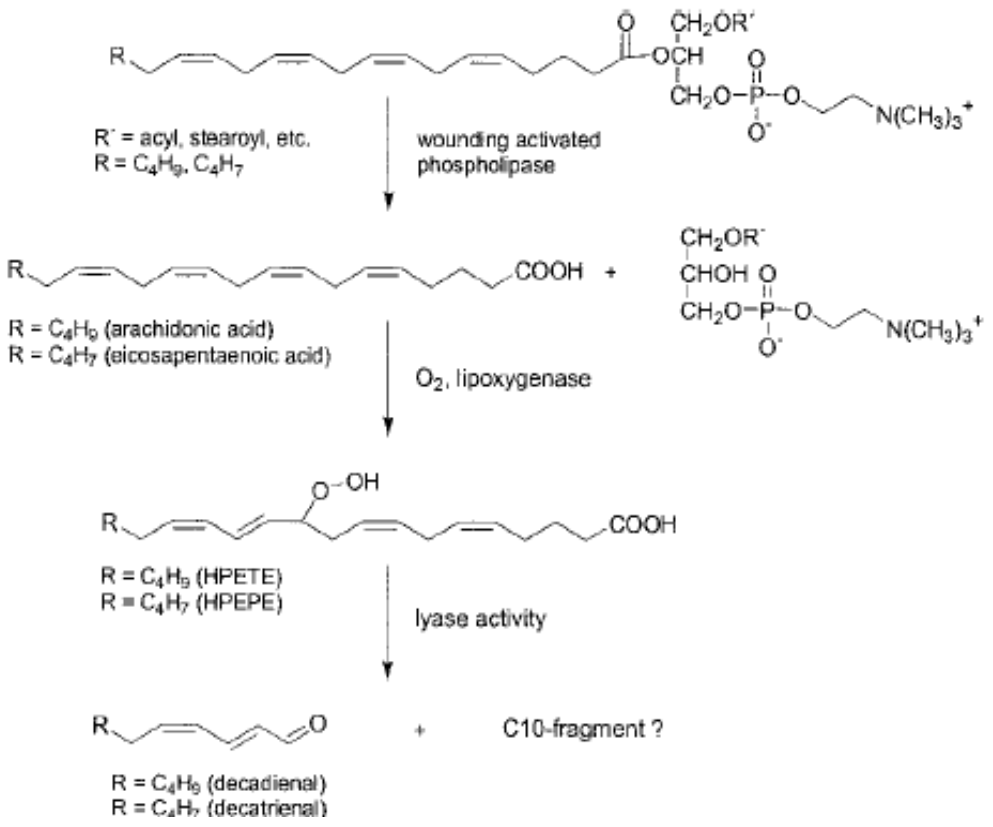


Figure 1.3 The proposed pathway for wound-activated transformation of eicosanoid fatty acids in *T. rotula* (Adapted from Pohnert, 2002).

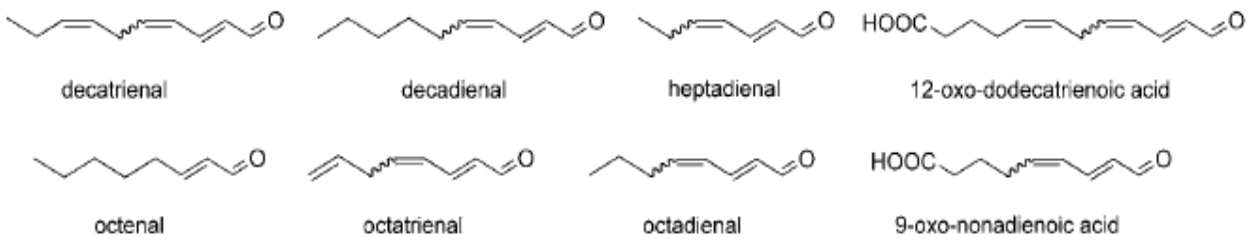


Figure 1.4: Various defensive metabolites from marine organisms (Pohnert 2002, 2005).

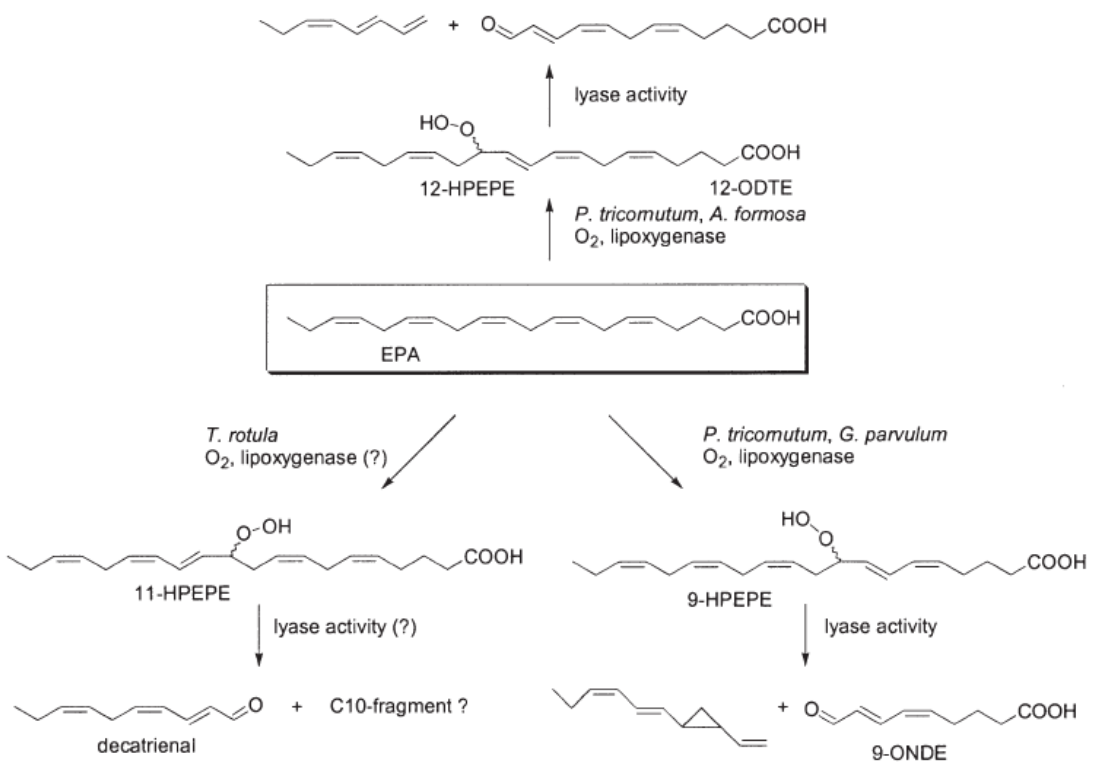


Figure 1.5: Production of various EPA derived oxygenated products via action of enzymes lipoxygenase/ hydroperoxide lyase in diatoms *P. tricornutum*, *A. formosa*, *G. parvulum* and *T. rotula* (Adapted from Pohnert, 2002)

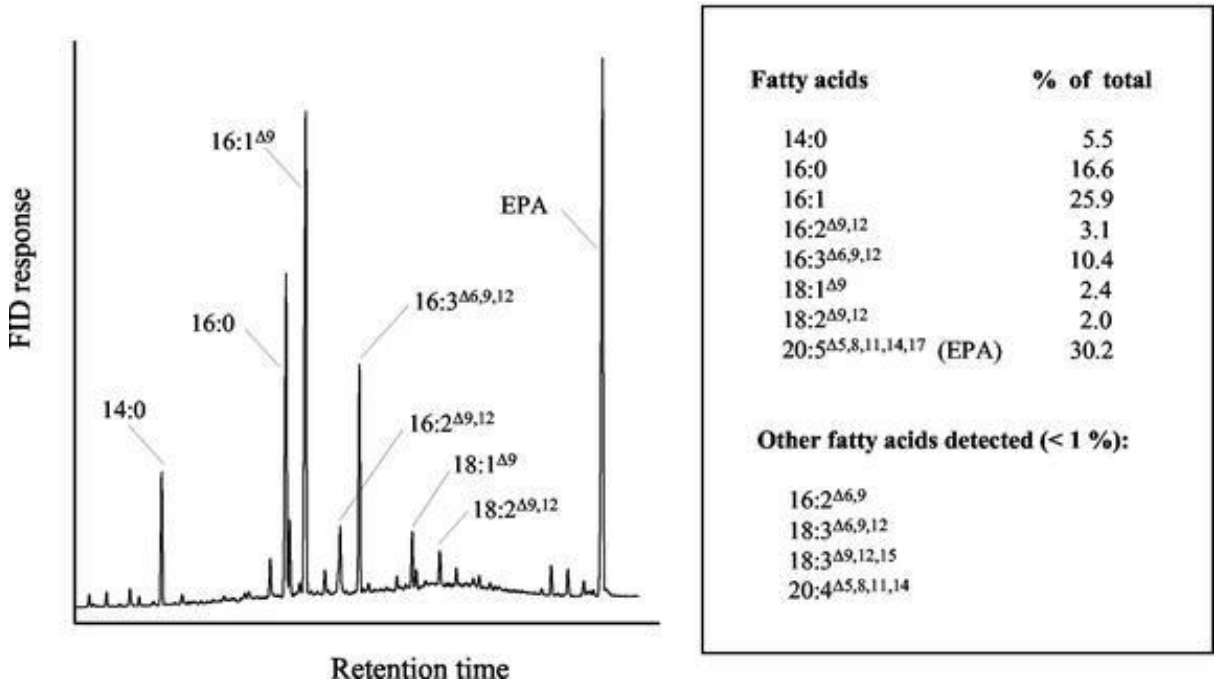


Figure 1.6: Fatty acid composition of *P. tricornutum* (UTEX 646) (Domergue et al., 2003).

Figure 1.7: Known possible biosynthesis routes of EPA synthesis in *P. tricornutum* (Domergue, 2002).

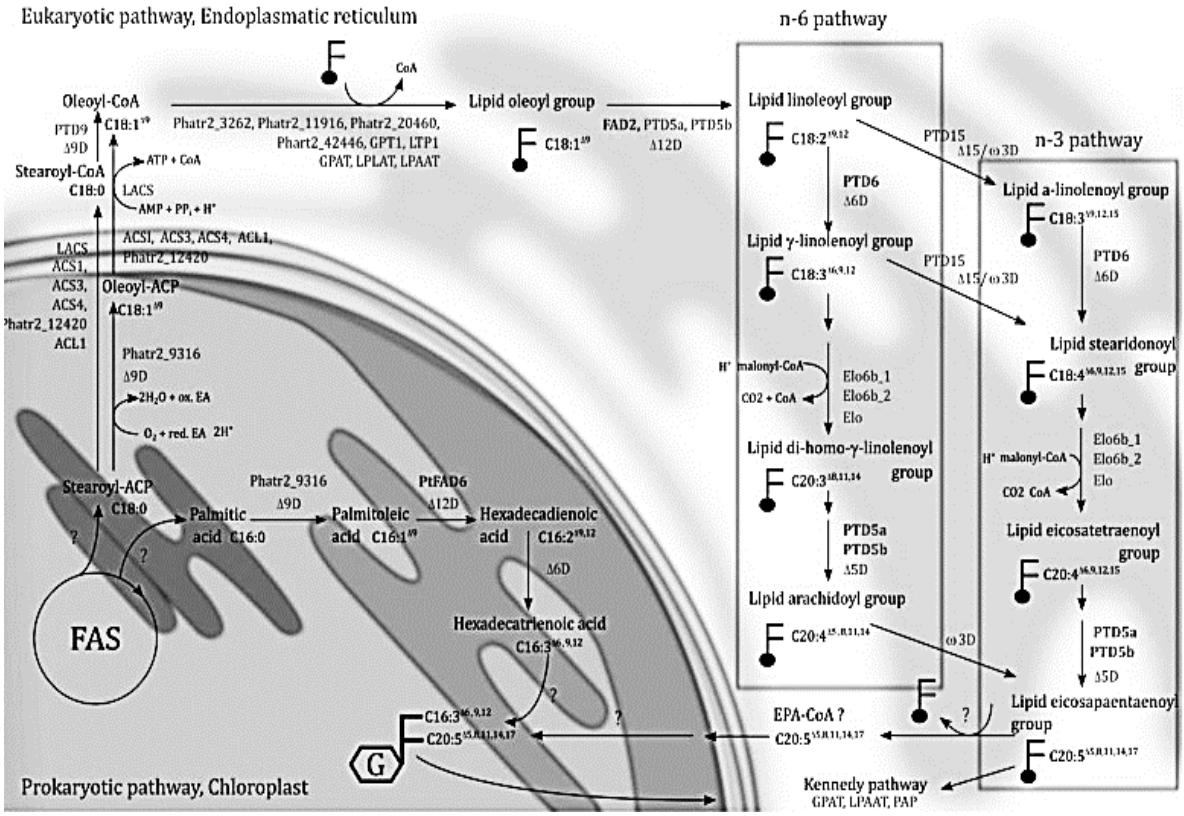


Figure 1.8: Known pathways for synthesis of saturated and unsaturated fatty acids in *P. tricornutum* with predicated genes involved in the pathways. (Adapted from Muhlroth et al., 2013).

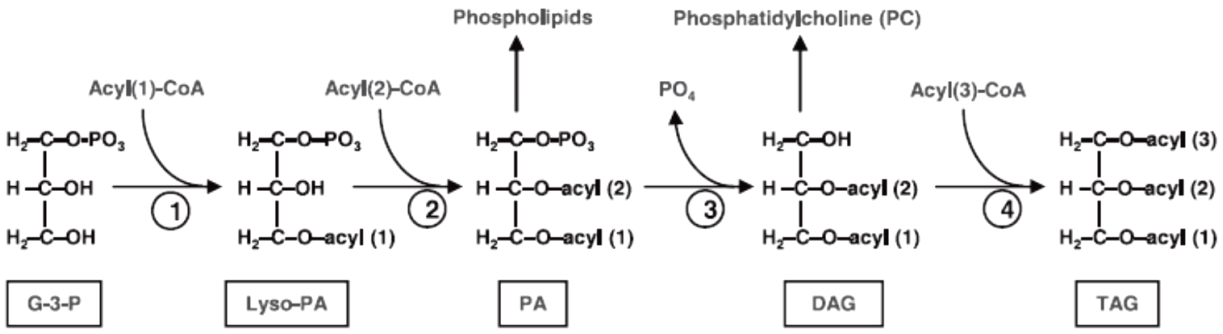


Figure 1.9: Known pathway for production of TAGs in algae. (1)Cytosolic glycerol-3-phosphate acyl transferase (GPAT), (2) lyso-phosphatidic acid acyl transferase (LPAAT), (3) phosphatidic acid phosphatase (PAP) and (4) diacylglycerol acyl transferase (DGAT). (Adapted from Hu et al., 2008).

Chapter 2: Defense-related decadienal elicits changes in fatty acid and lipid class composition in the diatom *Phaeodactylum tricornutum*[2]

Abstract

Many diatoms rapidly synthesize and release extracellular oxylipins (oxygenated lipids) including polyunsaturated aldehydes (PUAs) in response to herbivory and other stresses. PUAs have several defense related biological activities including inhibition of reproduction in herbivores and signaling to distant undamaged diatoms in the population. However, PUA effects on unstressed diatoms within a population are largely unknown. We applied the model PUA decadienal (DD) to unstressed *Phaeodactylum tricornutum* cultures to test the hypothesis that PUA exposure will result in modification of lipid pools and that plasma membrane permeability will decrease as part of defense- related responses.

Treatment of *P. tricornutum* with 10µM DD altered lipid compositions primarily by modification of existing lipid pools. In the total fatty acid pool of cells treated for 3 hr, nine out of ten measured saturated and unsaturated fatty acids declined (range of 0.5-0.7 fold of solvent control levels). In addition, phospholipids PG[3] and PS[3] declined (0.69 fold, 0.36 fold, respectively) whereas PE[3] increased (1.79 fold). At 6 hr, most fatty acid species 14:0, 18:0 and 20:5 remained at lower levels while 18:1 and 18:2 increased (1.12 fold and 1.46 fold respectively). Also, there were declines in PI[3] (0.79 fold), PS[3] (0.28 fold) and LPG[3] (0.56 fold) lipid classes and increases in PC[3] (1.21 fold) and PE[3] (2.7 fold). DD had no detectable effect on abundant glycolipids (DGDG/MGDG)[3]. Molecular species composition of phospholipids showed a greater decline in PUFAs[3] than MUFAs[3] or SFAs[3]

[2] This chapter after journal specific edits and formatting will be a manuscript for submission to journal.

[3] PG= phosphotidylglycerol; PS= phosphotidylserine; PE= phosphotidylethanolamine; PC= phosphotidylcholine; LPG= lysophosphotidylglycerol; PI=phosphotidylinositol; DGDG= digalactosyl diacylglycerol; MGDG= monogalactosyl diacylglycerol; PUFAs= polyunsaturated fatty acids; MUFAs= monounsaturated fatty acids; SFAs= saturated fatty acids

indicating a shift towards more saturated fatty acids in response to DD. C-28 brassicasterol declined 0.86 fold at 3 hr whereas there was no change at 6 hr. Non-polar lipids increased by 1.16 fold and 1.38 fold at 3 and 6 hr. Overall, the composition of 18:1, 18:2, PC, PE and neutral lipids increased while 14:0, 18:0, 16.2, 16:3, 20:5, 22:6, PI, PS, and LPG decreased. Changes in membrane permeability were reported as measured by decline in uptake of two out of three cellular dyes assayed in DD treated cells. We speculate the changes in lipid composition induced by DD may lead to synthesis of oxylipins to inhibit herbivores and to signal neighboring diatoms. In addition, the changes in membrane lipids may protect diatoms against oxidative stress and help in their ecological adaptation by altering membrane properties.

Introduction

Diatoms are ubiquitous microalgae responsible for about one fifth of photosynthesis on earth. Diatoms release bioactive compounds called oxylipins in response to stress such as mechanical cell lysis, cell damage by grazers, competition among species and unfavorable conditions such as nutrient limitation. Cell damage by grazers triggers production of PUFAs and polyunsaturated aldehydes (PUAs) in various marine and freshwater diatoms that acts as a chemical defense and protects phytoplankton populations (Pohnert G, 2005). One such PUA is Decadienal (DD) that has been shown to inhibit reproductive ability of copepods. Studies done by Pohnert (2002) and Wichard et al. (2007) showed that mechanical damage (sonication) to PUA producing diatoms such *as Thalossiosira rotula*, *Skeletonema coastatum* and *Skeletonema turris* resulted in decline of polyunsaturated fatty acids (mainly 20:5, 16:3 and 16:4) within 30 minutes whereas saturated fatty acids remained unchanged. The PUAs rapidly released in these diatoms were derived from the conversion of C_{16} and C_{20} PUFAs in polar or galactolipids. In contrast, non-PUA producing diatoms such as *Thalossiosira weisflogii* and *Thalossiosira pseudonana*, exhibited no change in saturated and unsaturated fatty acids content.

P. tricornutum is considered valuable as a model diatom owing to its sequenced genome and ease of growth in the laboratory to study molecular and biochemical mechanisms. *P. tricornutum* has a good lipid and high EPA content making it a potential candidate for commercial purposes (Domergue F, 2003; Chisti, 2007). A study done by Wichard et al. (2005) on PUA production (upon cell disruption) surveyed 51 species (71 isolates) of diatoms including four strains (isolates) of *P. tricornutum.* The four strains produced no detectable PUAs (Wichard T et.al, 2005). However Laabir et al. (1999) and Pohnert et al. (2002) reported that *P. tricornutum* inhibits the hatching success of copepod *Calanus helgolandicus* and on development of sea urchin eggs and blastulas. Pohnert and Boland (2002) reported that upon wounding, *P. tricornutum* released C11 homoserine and C8 fucoserratene hydrocarbons along with aldehydic acids 9-ONDE and 12 ODTE that were anti-proliferative similar to DD. While previous studies have reported lipid changes in mechanically damaged diatom cells, the effects of released PUAs or other oxylipins on lipid levels in distant undamaged diatoms are unknown.

There is considerable evidence that PUA released from stressed diatom cells participates in communication to unstressed cells. DD treatment of unstressed *P. tricornutum* cells results in calcium dependent NO production within 5 minutes and genome wide transcriptome changes measured within 6 hr. (Leflaive J et al., 2009; Vardi et al., 2006). The production of NO was found to be DD dose dependent between 33 µM-66 µM DD as observed by Vardi et al. (2006). Cell populations treated with DD concentrations (3.3µM-to 13.2µM) for 24 hr when mixed with population of cells unexposed to DD induced NO production in the unexposed cells. This indicated transmission of a DD induced diffusible signal from wounded to healthy cells but the nature of the signal was not elucidated. Lethal levels of DD (>3µg/ml = ~19.8 µM) induced programmed cell death (PCD) in dose and time dependent manner with about 90% of cell death at 66µM DD concentration in 4 hr. Diatom cells receiving sub-lethal levels of this aldehyde were resistant to subsequent exposure to lethal levels showing increased growth

and photosynthetic efficiency, suggesting a physiological defense related benefit which may enable cells to survive in areas of intense herbivory (Vardi et al., 2006, 2008).

It is important to understand how oxylipins released from the site of herbivory affects composition of lipids in healthy cells. Our data indicate oxylipins may serve as a potential defense mechanism against grazers, as a factor that may affect transfer efficiencies between primary producers and consumers, and as a regulator of diatom biomass for commercial products.

In this study, we determined the effects of decadienal on fatty acid levels and lipid classes in healthy *P. tricornutum* cells within a short time scale (6 hr). We hypothesized that DD induces changes in fatty acids in the membrane phospholipids. These changes might be related to downstream production of any oxylipin(s) and/or could be an adaptive way of reorganizing membrane lipids to cope with oxylipin stress. Also, we hypothesized that non-polar lipids may remain unchanged or possibly increase due to slowed photosynthesis to storage energy during decadienal stress.

Overall, we observed a shift towards saturated and monounsaturated species rather than polyunsaturated fatty acids, particularly in the phospholipids pool, and we documented changes in the phospholipid pool composition, as measured at 3 hr and 6 hr after DD exposure. These changes in predominantly membrane lipids may be a strategy to help cells stabilize membranes during DD stress and adapt to new environments with high herbivory.

Materials and methods

Strain and culture conditions

Phaeodactylum tricornutum (CCMP2561) was ordered from Provasoli-Guillard National Center for Culture of Marine phytoplankton (CCMP-http://serc.carleton.edu/resources/20043.html), USA and grown in f/2 medium made with steam-sterilized local seawater with f/2 vitamins (filter sterilized) and inorganic nutrients. Cultures were incubated under cool-white fluorescent lights at 80 μmol m^{-2} s^{-1} on a 12:12 hr light/dark cycle at 20 °C for about 10-12 days and periodically stirred by hand. Viability and NO measurements were done both at exponential phase (8x10^5 to 9x10^5 cells/ml) and stationary phase (2.5-3x10^6 cells/ml). For fatty acid and lipid measurement experiments cells were grown to stationary phase (2.5x10^6 to 3x10^6 cells/ml).
Cells were harvested by centrifugation for 10 min at 3500*g* for all experiments. Cell pellets were frozen instantly in liquid nitrogen and lyophilized and stored at −80 °C before for total lipid extraction and FAME preparations experiments. For all the experiments, cells were induced with treatment (decadienal, DD) at 3hours into light cycle.

Chemicals

Chemicals and Vitamin Stocks for preparing F/2 media for growing *P. tricornutum* cultures were obtained from UTEX culture collection, UT Austin. SNP (sodium nitroprusside; 100 mM fresh stock in water), Anhydrous DMSO and Decadienal (DD) were obtained from Sigma-Aldrich (W313505; http://www.sigma-aldrich.com). DAF-FM (5 mM stock in DMSO), Sytox Green nucleic acid stain (5 mM stock in DMSO) and BODIPY lipid stain 505/515 (10 mg powder) were purchased from Molecular Probes-Invitrogen (http://probes.invitrogen.com). Tri-Tri Decanoin (13:0 internal standard) was purchased from Nu Chek prep, Inc. All the chemicals and solvents used for fatty acid methyl ester preparation and total lipid extraction were either ACS or HPLC grade purchased from Fisher Scientific and were degassed before use. Evans blue (E2129-10G) was ordered from

Sigma-Aldrich and 1 % stock solution was prepared in water. Fluorescent dyes Hoechst 33342 (H3570; 10 mg/ml) and CellTrace CFSE (Carboxyfluorescein succinimidyl ester) stain (C34554) were purchased from Molecular probes-Thermo Fisher Scientific.

Growth assay

Growth assay was done to determine the effect of DD on cells. Cells were inoculated at 5x10^5 cells/ml conc. and were either incubated with DD or DMSO solvent (0.1%) control and flasks were grown over a period of 7 days into stationary phase. Cells were observed under microscope for normal morphology of cells and counts were done every alternate day using a hemocytometer to determine growth of cells.

Viability assay

Viability of cells were assayed by using a fluorescent sytox green nuclei stain to see an effect of DD concentrations over a period of 6 hr. Cells were treated with either water control, DMSO (0.1%) solvent control or DD (10 µM and 50 µM) and were harvested at 2 hr, 4 hr and 6 hr. Cells were re-suspended in 10 mM hepes buffer with 1 µM sytox stain and incubated for 10 minutes according to manufacturer's (molecular probes) protocol from where sytox stain was purchased. Cells were assayed on BD LSR Fortessa flow cytometer to determine % viability of cells over time.

Measuring NO production in cells

For NO measurements, *P. tricornutum* cells were incubated in the dark with 10 µM DAF-FM for 60 min followed by two washing steps (incubation for 30 min after the first wash to allow de-esterification). Efficiency of loading was tested by examining DAF-FM–dependent fluorescence in the microscope following addition of the NO donor SNP (0.5

mM). To quantify NO accumulation, DAF-FM fluorescence was measured using a BD LSR Fortessa flow cytometer equipped with a 488-nm laser as excitation source. A 530/30BP emission filter was used for detection of DAF-FM–derived fluorescence.

FAME preparation

Fatty acid methyl esters (FAMEs) were prepared directly from cell pellets using the protocol modified from Zhou et.al (2008). Cells were in their stationary phase (~2 to 3x10^6cells/ml) when harvested for preparing FAMEs and constant total number of cells (~1 to 1.5x10^8) and volumes were used for each preparation. Five biological replicates of DMSO (0.1%) solvent control and 6-8 biological replicates of 10µM DD treated samples at 3 hr and 6 hr were used. Two independent experiments for samples analyzed at 3 hr and three independent experiments for samples analyzed at 6 hr were done. Cell pellets were quickly flash frozen with liquid nitrogen after harvesting and then lyophilized for 3-4 hr and frozen in -80°C until use. Internal standard 13:0 (Tri Tri Decanoin) was added to each sample at fixed amount before starting FAME preparation. BHT (0.01%) was added to solvents while FAME preparation to minimize any losses due to oxidation of fatty acids. After FAME preparation, fixed volume of each sample (processed upper phase) was dried under nitrogen gas and sent on dry ice for GC-MS analysis at UT San Antonio.

GC/MS Analyses

Gas chromatography/mass spectrometry (GC/MS) analyses were performed on a ThermoFisher (San Jose, CA) TRACE DSQ single quadrupole mass spectrometer at Mass spec lab, University of Texas at San Antonio (http://research.uthscsa.edu/mass-spectrometry/). GC conditions were as follows: column, DB-225MS (Agilent; Santa Clara, CA), , 30 m x 0.25 mm, 25 µm film thickness; carrier gas, helium; linear velocity, 1

ml/min (constant flow); injection volume, 1 µl; injection, splitless, 50 ml/min split flow after 1 min; injector temperature, 220 °C; column temperature program, initial temperature of 50-°C held for 1 min followed by an increase to 200 °C at 50 °C/min then an increase to 240 °C at 10 °C/min. MS conditions were: ionization, EI (70 eV); detection, positive ion; full scan analyses, m/z 50 – m/z 400 at two scans/sec. Peak areas were obtained by manual integration of total ion current chromatograms, using Xcalibur (ThermoFisher). Each fatty acid peak in the GC chromatogram was identified based on the GLC-85 reference standard mix. Then area was determined for each peak and relative proportion for each peak was determined by dividing the area of the peak by the area of internal standard. Fold changes in specific FA were determined by dividing the relative proportion of certain FA in DD treated samples with relative proportion of that FA in DMSO solvent control.

ESI-MS/MS analysis of lipid molecular species

Cells (~1.4x10^8 cells) were treated with DMSO (0.1%) solvent control and 10µM DD treated cells at 3hr and 6hr and then harvested. Pellet was flash frozen with liquid nitrogen, lyophilized and then stored in -80^{o}C until lipid extraction. Total lipids were extracted from frozen lyophilized *P tricornutum* cells using a procedure modified from Welti et.al (2002). To the lyophilized pellet, 2 ml of chloroform and 4 ml of methanol was added, tubes were vortexed to mix and then 2 ml chloroform and 2 ml water was added. Tubes were vortexed and then centrifuged at 3500 rpm for 10 minutes. Lower phase was collected in clean glass tube. 2 ml chloroform was added, vortexed and centrifuged and lower phase was collected. Lower phases were combined and washed once with 0.5 ml KCL and once with 0.5 ml water. Extracted lipids samples were dried under nitrogen and sent for ESI-MS/MS analysis at the Analytical Laboratory of the Kansas Lipidomics Research Center (https://www.k-state.edu/lipid/).

Sterol Extraction and GC-MS Analysis

Total lipids were extracted from 1.5x10^8 cells from 4 replicates of DMSO solvent control cells and 5 replicates of 10 µM DD treated cells at 3 hr and 6 hr using the protocol as described earlier (modified from Welti et.al, 2002). Extracted lipids were completely dried under nitrogen gas and shipped to Kansas State University (KSU) lipidomics center where samples were further processed. Each sample was re-dissolved in 1 ml chloroform and 700µl was used for saponification. 4 µL of internal standard-cholestanol (Stock solution: 1mg in 1 mL of chloroform, Steraloids, Inc) and dried down under nitrogen. Then, 1 mL of methanolic potassium hydroxide (3 M KOH: MeOH v/v 1:9) was added and heated at 80°C for 1 h. After cooling down, 2 mL of HPLC-grade water was added and the sterols were recovered by extracting the mixture 3 times with 2 mL hexane. The hexane extracts were pooled and dried down under nitrogen. Each dried extract was re-dissolved in 70 mL pyridine and 30 µL N-trimethylsilyl-N-methyltrifluoroacetamide with trimethylchlorosilane was added and incubated at 50 °C for 60 min. Each sample extract was dried completely under nitrogen and re-dissolved in 100 µL hexane. For analysis 1µl of each sample was injected to GC-MS.

GC Method: GC-MS was performed on an Agilent 6890N GC coupled to an Agilent 5975N quadrupole mass selective detector. The GC was fitted with a DB-5MS capillary column with a 5% phenyl, 95% methylpolysiloxane stationary phase (column length: 60 m, internal diameter: 250 µm, film thickness: 0.25 µm). Helium was used as the carrier gas at a column flow rate of 1 mL/min. The front inlet was operating at a pressure of 24.93 psi and 280 °C. The Agilent 7683 autosampler was used to inject 3 µL of the sample in the splitless mode. The GC oven temperature program was: initial temperature of 150 °C, hold 1 min. increase 30 °C/min to 300 °C, then increase 3 °C/min to a final temperature of 315 °C, hold 5min. Total run time was 16.00 min. The mass spectrometer was operated in the electron impact mode at 70 eV ionization energy. The MS quad temperature was at 150 °C and the MS

source temperature was at 230 °C. The data acquisition was at scan mode. Scan mass was 50 to 650. The data were processed with Agilent Chemstation software.

Measuring neutral lipids in P. tricornutum cells by Bodipy

For intracellular neutral lipids determination, the fluorescence dye, BODIPY 505/515, was diluted in anhydrous DMSO to achieve a final concentration of 0.5 mg/ml as the stock solution. Water control, DMSO (0.1%) solvent control and 10 μM DD treated cells harvested and resuspended in hepes buffer with a final concentration of 0.075 μg/ml bodipy dye and incubated for 10 minutes at room temperature. The protocol for staining cells with bodipy was adapted and modified from Govender T et al (2012) and that recommended by manufacturer molecular probes from where dye was purchased. Cells were assayed within 30 minutes of dye addition on BD Accuri flow cytometer (equipped with 488 nm laser) to quantify lipid based fluorescence.

PUA analysis

For rapid screening and analysis of volatiles, solid phase microextraction was performed with a polydimethylsiloxan fiber using the procedure modified from that described in Pohnert et al. (2002). 50 ml of a DMSO (0.1%) solvent control and 10μM DD treated cultures (3 hr and 6 hr time points) in the stationary phase (~2.8x10^6 cells/ml) were concentrated by centrifugation and resuspended in fresh 3 ml media in vials which were sealed using a Teflon cap. A polydimethylsiloxane-coated (100 μm) SPME fiber (SUPELCO) was introduced in the headspace over the medium. Extraction was performed for 15 mins at 50°C with continuous stirring of culture. Evaporation of the analytes from the fiber was directly performed within the injection port (220 °C) of the GC-MS (DB225-MS column, Alltech; T-program: 50 °C [2 min, splitless]; ramped with 10 °C min–1 to 200 °C and with 30 °C min–1 to 280 °C [2 min]). Decadienal standard (10 μM mixed in media) was also run as a control.

Membrane permeability assays

Three different stains were evaluated for their permeability into DMSO (0.1%) solvent control or 10µM DD treated cells at 3 hr and 6 hr. Evans blue (visible stain) and Fluorescent cell-permeant stains Hoechst 33342 and CFSE (Carboxyfluorescein succinimidyl ester) stains were applied and data was analyzed for permeability differences between DMSO control and DD treated cells for 3 independent experiment trials (with 3-5 replicates for control and treated type at each time point). Staining of cells with Evans blue was done using the procedure described in Garrison et. al (2014). Using 1% stock solution, dye was added to cells at final concentration of 1:50 dilution and incubated for 15 minutes before counting on hemocytometer. Counting was done in triplicates and average was taken for final count of stained cells. Data was expressed as % stained cells and statistical significance was calculated by two sample unpaired independent student's t-test.

For staining cells with Hoechst 33342, manufacturer's recommended procedure was followed. Final concentration of stain was added to cells at 1:2,000 dilutions to cells in hepes buffer and incubated for at least 10 minutes in dark before assaying on Zeiss Axiovert 200M fluorescent microscope and BD FACSAria flow cytometer with DAPI filter set (Ex 350/ Em 461). Staining cells with CFSE dye were as described in manufacturer's protocol. Stock solution of CFSE (5 mM in DMSO) stain was added at 5 µM final concentration to cells in hepes buffer and incubated for 20 minutes in dark at room temperature. Five times original staining volume of Hepes buffer was added to the cells and incubated for 5 minutes. Cells were washed and resuspended in fresh buffer and incubated for at least 10 minutes before imaging at Zeiss Axiovert 200M fluorescent microscope and assaying on BD Acurri flow cytometer with FITC filter set (Ex 492/ Em 517). Microscope images were processed using Fiji (Image J) and flow cytometer data was analyzed to get median fluorescence intensity (MFI) of solvent control and DD treated cells. Statistical significance was calculated using two sample unpaired independent student's t-test.

Results and Discussion

Growth and viability assay to determine effect of DD on cells

To determine efficacy and optimize dose of DD in our cellular system, growth and viability assays were done. DD affected growth and viability in a dose and time dependent manner. Concentrations of DD above 20µM were detrimental on cells whereas 10µM DD slowed growth, but cells continued to grow for 7 days (supplementary figure 2.7). Based on cell absorbencies at 680nm no significant change in growth over 0-6 hr was observed for 10 and 20µM DD treated cells compared to DMSO solvent control (data not shown). Viability assays done on cells both at exponential and stationary phase on flow cytometer using sytox green fluorescent dye showed that there was 13.4 fold increase in fluorescence (indicating compromised cells) by 6 hr for 50µM DD concentration whereas for cells treated with 10µM DD increase in fluorescence by 6 hr was 1.2 fold when compared to DMSO (0.1%) solvent control for cells in exponential phase (supplementary figure 2.8). At stationary phase for cells treated with 10µM DD, increase in fluorescence was 1.05 fold and for that treated with 50µM DD was 4.4 fold by 6 hr compared to DMSO (0.1%) solvent control (Figure 2.1).

Based on cell growth and viability experiments, 10µM DD dose was chosen to be optimal to use for subsequent experiments. This DD dose is also close to the physiologically relevant dose that induces reproductive failure in copepods and arrest copepod larval development as reported by previous studies (Pohnert, 2002; Ianora et al., 2004, 2010; Leflaive and Ten Hage, 2009).

DD dose and time dependent NO production in the cells

To further validate the effect of DD in our system, NO production was measured. Previously Vardi et al (2006) have shown that NO production in *P tricornutum* cells in response to DD was dose and time dependent. They showed an increase in NO production

with 33, 46 and 66 µM of DD within 4 hr time point. Our results also showed a similar correlation of DD dose and time with NO production in *P tricornutum* cells. There was 1.95 fold and 8.92 fold increase in NO fluorescence with 10 µM DD and 50 µM DD, respectively, by 6 hr for cells in exponential phase (supplementary figure 2.9). For cells in stationary phase, there was 1.62 fold and 3.25 fold increase in NO production by 6 hr with 10 µM and 50 µM DD treatment (Figure 2.2).

Effect of DD on changes in fatty acid levels in P. tricornutum

The major fatty acids peaks detected and identified in *P. tricornutum* were 14:0, 16:0, 18:0, 16:1, 16:2, 16:3, 18:1, 18:2, 20:5, 22:6 which agreed with previously reported fatty acids in *P. tricornutum* (Domergue, 2003) (supplementary figure 2.10). Effect of 10µM DD was analyzed on measured saturated and unsaturated fatty acids by determining fold changes in the fatty acids in DD treated samples compared to DMSO solvent control at 3 hr and 6 hr (Table 2.1).

The data from Table 2.1 shows that 10 µM DD had an effect on various measured saturated and unsaturated fatty acids measured at 3 hr and 6 hr time points. At 3 hr with DD treatment, there was a significant ($p<=0.05$) decline in 14:0 (0.68 fold), 18:0 (0.57 fold), 16:2 (0.48-0.52 fold), 16:3 (0.57 fold) and 22:6 (0.52 fold). There was also a significant ($p<=0.1$) decline in 16:0 (0.69 fold), 16:1 (0.62 fold), 18:2 (0.66 fold) and 20:5 (0.56 fold). Changes in 18:1 were not significant. At 6 hr with DD treatment there was a significant ($p<=0.05$) decline in 14:0 (0.81 fold), 18:0 (0.74 fold), 16:2 (0.84-0.73 fold), 16:3 (0.80 fold), 20:5 (0.82 fold) and 22:6 (0.81 fold). However fatty acids 18:1 and 18:2 showed a significant ($p<=0.05$) increase by 1.12 and 1.46 fold. No significant changes were observed in levels of 16:0 and 16:1 fatty acids. A possible reason of decline in certain saturated fatty acids could be its conversion to other unsaturated fatty acids or other secondary metabolites by action of various enzymes in fatty acid synthesis pathway. A decline in 18:0 could be attributed to increase in 18:1 and 18:2 fatty acids which might be

due to DD induced increased activity of delta 9 desaturase enzyme. In general it was seen that there was greater decline in polyunsaturated species than monounsaturated and saturated species indicating shift of fatty acid pools towards more saturated or monounsaturated species. Fold changes in (SFAs+MUFAs) were 0.65 and that in PUFAs were 0.55 at 3 hr and 0.94 and 0.87 at 6 hr in DD treated cells compared to DMSO solvent control (Table 2.2) indicating increased proportion of saturated and monounsaturated fatty acids over polyunsaturated fatty acids. Since polyunsaturated fatty acids are more prone to be damaged during oxidative stress, this DD induced shift might help protect fatty acids in cells from oxidative/NO stress. Also for utilization of biomass in lipid extraction for biofuel or second generation fuels saturated and monounsaturated fatty acids are more desirable due to PUFAs being more prone to oxidative attack (Schenk et al., 2008).

Interestingly studies done by Kumar et al. (2010a) on salinity induced oxidative stress in red alga *Gracilaria Corticata* showed similar increases (measured within 24 hr) in 18:1, 18:2 and 18:3 fatty acids (1.3-1.6 fold) with decrease in 16:1 during hypersalinity conditions (>35), which they suggested was due to increased delta 9 desaturase activity responsible for converting 18:0 to unsaturated C18's. Authors also suggested that this could be an adaptive strategy for maintaining the requisite greater membrane fluidity, to stabilize the protein complexes of photosystem II and to control membrane physicochemical properties, such as the increased activity of Na+/H+ antiport system of the plasma membrane to cope with hyper-salinity stress. In another study, on cadmium induced oxidative stress, the marine algae *Ulva lactuca* showed similar results, wherein 2-fold increases in 18:2 and 18:3 fatty acid at the expense of 16:0 and 16:1 fatty acids were reported (measured after 96 hours), suggesting that level of unsaturation of lipids could be required to maintain the degree of fluidity needed for proper functioning of membranes and membrane bound enzymes (Kumar et al., 2010b). These data suggest that in DD induced stress unsaturated C18 fatty acids might be playing a role to maintain proper functioning of membranes and cope with oxidative stress. Wichard (2007) reported that

unsaturated C16s and 20:5 act as substrates for production on downstream oxylipin. In our case, decline in unsaturated C16s and 20:5 may also be related to DD induced production of downstream products (oxylipins). Initial attempts using the SPME technique (as described in Pohnert, 2002) could not identify any volatile compounds produced by DD treated *P. tricornutum* cells at 3 hr and 6 hr (data not shown). This agrees with earlier reports of Wichard et al. (2005) who could not detect volatiles from any of the four strains of sonicated *P. tricornutum* cells. It could be that DD induced *P. tricornutum* cells do not make any downstream volatiles or the amount they make is below the detection limit of the SPME technique. However Pohnert and Boland (2002) did report enzymatic production of aldehydic acids 9-ONDE and 12 ODTE from 20:5 precursor fatty acid from sonicated *P. tricornutum* cells. Further studies are needed to check whether DD induced *P tricornutum* cells might be making these aldehydic acids.

Fatty acids such as 18:2 and 18:3 are known to be involved in defense against pathogens in plants and in certain red and brown algae (Kupper et al., 2009). In Arabidopsis 18:2 and 18:3 induce ROS production by acting as activators of NADPH oxidase (Yaeno et al., 2004). Homologs of NADPH oxidase have been identified in certain red algae and diatoms *P. tricornutum* and *Thalassiosira pseudonana* (Herve et al., 2006). Since DD is known to induce NO production in *P. tricornutum* (Vardi et al., 2007), increases in C18 unsaturated fatty acid might be related to NADPH oxidase mediated NO production and regulation of defense mechanisms. There is also a possibility of chemical or enzymatic oxidation of these unsaturated C18 fatty acids to cyclopentanones or other biologically active oxylipin production as studied in certain red and brown algae such as *C. crispus* and *Laminaria* (Kupper et al., 2009). Recent studies by Mandal et al. (2012) have shown that 18:1 is associated with NO regulation by binding to and regulating the stability of NOA1 (nitric oxide associated) protein levels in Arabidopsis. They showed that reduction in 18:1 levels led to increased accumulation of NOA1 and increased levels of NO in chloroplasts. Vardi et al. (2006) have shown that in *P. tricornutum* DD induces NO production and

overexpression of NOA1 increases NO accumulation. Hence it is possible that DD induced increase in 18:1 fatty acid levels regulates NO production and stability in a similar manner as observed in Arabidopsis plant.

These reported effects of DD on various fatty acid levels also help inform current efforts to evaluate utilization of the biomass of *P. tricornutum* and other similar marine microalgae for production of biofuels and other essential PUFAs important in human nutrition such as 18:1, 18:2, 18:3, 20:5 (EPA) and 22:6 (DHA) during the time of toxic blooms. Also the observed DD induced significant decline in 20:5 and 22:6 fatty acids (especially 20:5 being an abundant fatty acid) both at 3 hr and 6 hr might be beneficial for *P. tricornutum,* making them less desirable as preference, since grazers depend mainly on these fatty acids for their growth and reproduction (Ianora et al., 2010).

ESI-MS/MS analysis of various lipid classes in DMSO solvent and DD treated P. tricornutum cells

Lipid profiling using ESI-MS/MS involves direct infusion of lipid extracts into mass spectrometer to identify and quantify various lipid classes. Information on phospholipids and glycolipids speciated to level of head group and number of carbon atoms and double bonds present in acyl chains can be obtained. Present analysis identifies major galactolipids classes [monogalactosyldiacylglycerol (MGDG) and digalactosyldiacyclglycerol (DGDG)], major phospholipid classes [phosphotidylcholine (PC), phopshotidylethanolamine (PE), phosphotidylglycerol (PG) and phopsphotidylinositol (PI)] and other minor classes [phosphatidylserine (PS), phosphatidic acid (PA), lysophophatidylcholine (Lyso-PC or LPC), lysophosphatidylethanolamine (Lyso-PE or LPE) and lysophosphatidylglycerol (lyso-PG or LPG). The signals of the peaks of various lipid species in the samples were identified and quantified by comparing it to signals for peaks of internal standards added in known amounts (supplementary figures 2.11, 2.12,

2.13). From the total signal (100%), % of total signal for each lipid class was determined. The data obtained was analyzed and represented as mol% of each class (as signal of 1nmol of internal standard = signal of 1nmol of analyte of interest) (Table 2.3). Fold changes in lipid classes were calculated in DD treated cells compared to DMSO solvent control (Table 2.4).

In *P. tricornutum,* galactolipids MGDG (44%) and DGDG (14%) are most abundant lipids following various phospholipids such as PC (22%), LPC (1.5 %), PG (3.7%), PI (3.7%), PE (0.2%) and LPE (0.1%) as presented in Table 2.3. Overall it is interesting to note that DD doesn't affect galactolipids but mostly membrane phospholipids. Galactolipids are mostly part of chloroplast membranes indicating that DD doesn't interfere with chloroplast/thylakoid membranes within studied time scale (6 hr) but mainly affects plasma and ER membrane lipids. Table 2.3 and 2.4 shows that there were no significant changes in galactolipids (DGDG and MGDG) in response to DD at 3 hr and 6 hr which constitute about 58% of total measured polar lipids. Certain classes of phospholipids had a significant effect of DD treatment. At 3 hr with DD treatment there was a significant decline in PG and PS lipids by 0.69 and 0.36 fold and a significant increase in PE by 1.8 fold. At 6 hr with DD treatment there was significant increase in PC and PE levels by 1.21 and 2.70 fold whereas significant decline in PS, PI and LPG levels by 0.28, 0.78 and 0.56 fold (Tables 2.3, 2.4). There was prominent increase in LPE by 1.24 and 1.32 fold at 3h and 6h but changes were not significant due to variation in one of the five biological replicates. Similarly there was a prominent increase in PA by 1.58 fold at 3h and decline by 0.77 fold at 6 hr but changes weren't significant due to variation among replicates owing to its low abundance (low signal) as compared to other lipids in the organism.

Lipid classes such as PC and PE maintain integrity and architecture of membranes and hence its function in various unicellular organisms, higher land plants and animals (Gibellini and Smith, 2010; Christie W- AOCS Lipid library). Studies done on unicellular yeast (Kroon et al., 2013) have shown that PC and PE are essential phospholipids in membrane determining

its physical properties. Depletion of PE in mitochondria results in impaired respiration and is also involved in delivery of cytoplasmic proteins to vacuoles by autophagy and that of transport of amino acid transporters to plasma membrane. PC is structurally similar to PE but the smaller headgroup of PE compared to PC results in tighter packing of acyl chains in PE bilayer. PC importantly forms stable matrix for (intra) cellular membranes stabilizing membrane bilayer. Previous study by Trombetta et al. (2002) has shown that 2-alkenals including DD can elicit a gross perturbation of lipidic fraction of plasmatic membranes, altering the membrane permeability and are able to penetrate in bacterial cells causing leakage of intracellular materials. Hence observed DD induced increases in PC and PE levels at 6 hr in *P. tricornutum* could be a way of reorganizing membrane lipids to maintain membrane stability, strength and permeability to maintain its normal biological function.

The Kennedy pathway for synthesis of PE and PC in mammalian, yeast, plant and algae cells is well studied. Plants and algae can synthesize de novo ethanolamine from serine via soluble decarboxylase. However an indirect route is known to exist wherein PS can be decarboxylated to form PE (Gibellini and Smith, 2010). In this study the significant decline in PS and significant increases in PE both at 3 hr and 6 hr might indicate DD induced activation of PS decarboxylases to synthesize PE from PS in *P. tricornutum*.

Some studies in plants have shown that PC and PE help regulate drought, osmotic and cold stress in plants. Studies done by Tasseva G et al. (2004) in Arabidopsis show that PC synthesis is accelerated in both salt- and cold-treated plants within 24 hours. These changes are regulated by changes in expression of PC pathway genes. Although PC can be synthesized in plants by a combination of both CDP-Cho and methylation pathways, the authors showed that enhanced synthesis of PC triggered by salt and cold stress is due to an accelerated CDP-Cho pathway, and does not involve the methylation pathway. They also showed that PC and PE levels in salt stress were regulated by transcript levels of gene families involved in the PC and the interconnected PE biosynthesis. Transcript levels of gene family choline kinase (CKs) and phosphocholine cytidylyltransferase (CCTs) genes

are oppositely regulated with respect to these abiotic treatments. Hence it is possible that *P. tricornutum* regulates membrane PC and PE levels transcriptionally via CKs and CCTs during DD stress, in a manner similar to salt and cold stresses, to maintain membrane homeostasis during stress. To our knowledge, this is the first report documenting novel rapid (within 6 hr) phospholipid changes in response to PUA suggesting early membrane lipid remodeling to aid in adaptation to PUA stress in algae.

Recent studies on phospholipid metabolism in *C. sorokianana* have shown that changes in membrane phospholipids can have an impact on use of its biomass for overall production and productivity of biofuels and other secondary metabolites (Lu S, Wang J et al, 2013). Hence decadienal induced lipid changes help determine industrial use of biomass of these organisms.

Further analyses of lipid molecular species were done to get more insight on changes in saturated and unsaturated fatty acids within phospholipid classes in response to DD.

Analysis of effect of DD on lipid molecular species within membrane phospholipid classes

ESI-MS/MS technique of lipid profiling further helps us get more insight on how various lipid molecular species are affected within the phospholipid classes. Polar lipid class (membrane lipids) are defined by specific head group or polar moiety and each class is composed of various molecular species whose fatty acids or other hydrocarbon positions vary in chain length and degree of unsaturation. This technique helps identify the head group apart from total fatty acids (both carbon length and degree of unsaturation). Fold changes in various lipid molecular species (Total carbon length and unsaturation level) within lipid classes PC, LPC, PE, LPE were determined in DD treated cells compared to DMSO solvent control (Table 2.5-2.8). Due to low abundance of PI, PS and PA lipid classes compared to others, many of the molecular species within these were either not detected or had very low signal and hence some variation among biological replicates.

Molecular species analysis of PE, PC, Lyso PE and Lyso PC lipid classes

Overall PE levels showed a significant increase of 1.79 fold ($p<=0.05$) at 3 hr with DD treatment. Measured molecular species in PE (Table 2.5) were mostly unsaturated species from C32 to 42 (double bonds 1 to 11) except saturated 32:0. Saturated 32:0 increased by 2.9 fold at 3 hr and declined to 0.4 fold at 6 hr with DD treatment but changes were not significant (Table 2.5). Unsaturated species such as 36:1and 36:2 showed significant increases in fatty acids levels by 10.48 fold and 5.94 fold ($p<=0.05$) at 3 hr and by 10.32 fold ($p<=0.1$) and 5 fold at 6 hr of DD treatment (Table 2.5).

Other unsaturated fatty acids such as 32:1, 32:2, 34:2, 34:3, 36:5, 36:6, 38:6,38:7,38:8, 40:9 and 42:11 showed significant increases ($p<=0.05$ except for 34:2 where $p<=0.1$) between 1.5-3.6 fold at 3 hr after DD treatment (Table 2.5). At 6 hr with DD treatment, 32:1 and 38:6 showed significant ($p<=0.05$) increase by 3.7 and 8.2 fold and other fatty acids such as 34:1, 34:2, 36:4,36:5, 38:7,38:8 showed a significant ($p<=0.1$) increase in between 1.3-3.1 fold (Table 2.5). It can be noted that in fold increases in saturated or lower unsaturated molecular species is higher than that of highly unsaturated species. Fold changes in SFAs+MUFAs were 2.33 and 3.35 whereas in PUFAs were 1.77 and 2.68 at 3 hr and 6 hr (Table 2.9) indicates increased proportion of saturated and monounsaturated species in response to DD treatment. Since both PE and PC are membrane lipids, these changes might be a possible strategy to prepare membrane against oxidative damage/stress as saturated or monounsaturated fatty acids are less prone to oxidation than polyunsaturated fatty acids.

Overall PC levels didn't show any significant change with DD treatment at 3 hr but significant increase of 1.21 fold at 6 hr. Further analysis was done to determine effect of DD on various molecular species which ranged from unsaturated C30 to 44 (double bonds -1 to12) and saturated 30:0 and 32:0 (Table 2.6). No significant change was seen in levels of 30:0 at 3 hr and 6 hr (Table 2.6). There was a significant increase in levels of 32:0 (1.54 fold; $p<=0.05$) at 3 hr (Table 2.6). At 6 hr 32:0 showed an increase in 1.22 fold but it was not found to be significant (Table 2.6). Among unsaturated species which showed a significant ($p<=0.05$) increase at 3 hr with DD treatment were 32:1(1.18 fold), 34:1 (3.20 fold), 34:2 (1.24 fold), 36:4 (1.41 fold), 38:4 (1.92 fold), 38:6 (2.32 fold), 40:4 (1.31 fold), 40:7 (1.46 fold), 42:3 (1.18 fold) and 44:12 (1.21 fold) (Table 3.2). Some other unsaturated species showed a significant decline such 34:3(0.86 fold), 34:4(0.76 fold), 34:5 (0.85 fold), 36:7 (0.76 fold), 38:7 (0.88 fold), 38:8 (0.66 fold), 38:9 (0.56 fold), 40:9 (0.65 fold) and 40:10 (0.86) (Table 2.6). At 6 hr with DD treatment significant ($p<=0.05$) increases in unsaturated species such as 32:1 (1.28 fold), 34:2 (1.50 fold), 34:1(3.71 fold), 36:3 (1.34 fold), 36:5 (1.17 fold), 38:4(2.45 fold), 38:6(4.13 fold), 38:7 (1.17 fold), 40:7 (1.67 fold), 40:8 (1.30 fold) and 42:11 (1.13 fold) were seen (Table 2.6). Other unsaturated species that showed a significant ($p<=0.05$) decline were 32:4 (0.83 fold), 34:4 (0.72 fold), 34:5 (0.77 fold), 36:7 (0.76 fold), 38:5(0.28 fold), 38:9(0.44 fold), and 40:9(0.62 fold) (Table 2.6). Overall there was a decline in highly unsaturated species and an increase in saturated species and low unsaturated species, which might help make cell membranes less prone to oxidative attack. The fold changes in SFAs+MUFAs were 1.45 and 1.57 whereas in PUFAs were 1.01 and 1.17 at 3 hr and 6 hr (Table 2.9) with DD treatment indicating abundance of saturated and monounsaturated fatty acids over polyunsaturated fatty acids.

Changes in LPE levels at 3 hr and 6 hr were not found to be significant with 3 hr and 6 hr DD treatment. Analysis of molecular species shows that only 18:1 showed a significant ($p<=0.05$) increase by 3.23 fold at 6 hr with DD treatment that agrees with previous fatty acid analysis data (Table 2.7). Fold changes in SFAs+MUFAs were 1.47 and 1.76 whereas

in PUFAs were 1.13 and 1.04 at 3 hr and 6 hr (Table 2.9) indicating overall more proportion of saturated and monounsaturated species over polyunsaturated species in membrane.

Overall changes in LPC were not found to be significant with DD treatment at 3 hr and 6 hr. However certain molecular species showed significant changes. Significant ($p<=0.05$) decline in levels of 18:3 (0.75 fold at 3 hr and 0.55 fold at 6 hr) and 20:5 (0.81 fold at 3 hr and 0.62 fold at 6 hr) were seen whereas significant ($p<=0.05$) increases in levels of 18:1 were seen by 2.46 fold at 3h and 1.97 fold at 6 hr (Table 2.8). Also at 3 hr significant ($p<=0.05$) decline in 18:2 by 0.86 fold was seen (Table 2.8). At 6 hr significant ($p<=0.05$) decline in levels of 18:0 were seen by 0.39 fold and in 16:1 by 0.77 fold ($p<=0.1$) (Table 2.8). Fold changes in total saturated and monounsaturated fatty acids were 1.41 and 1.03 and polyunsaturated fatty acids were 0.86 and 0.69 at 3 hr and 6 hr with DD treatment (Table 2.9) indicating abundance in SFAs+MUFAs compared to PUFAs for this lipid class.

Changes in LPG were not found to be significant at 3 hr with DD treatment whereas a significant ($p<=0.1$) decline by 0.56 fold was seen at 6 hr with DD treatment (data not shown). Molecular species analysis showed an increase in 18:1 and 18:2 fatty acids by 2.32 fold and 1.95 fold at 3 hr of DD treatment but changes were not found to be significant (data not shown). At 6 hr with DD treatment there was a significant decline in 16:0 and 16:1 fatty acids by 0.57 fold ($p<=0.05$) and 0.46 fold ($p<=0.1$) (data not shown).

Among other lipid classes, PI showed at significant ($p<=0.1$) decline at 6 hr whereas no change at 3 hr with DD treatment. Analysis of molecular species showed that there was a significant ($p<=0.05$) increase levels of 34:3 by 1.71 fold and decline in levels of 32:1 by 0.77 fold ($p<=0.1$) (data not shown). Most molecular species in this class ranged from C32 to 36 and low unsaturated species (double bonds 1 to 5) unlike other classes that had very highly unsaturated species. This supports changes seen with respect to proportion of

saturated and less unsaturated species to highly unsaturated species to maintain stability in membrane lipids.

PS showed a significant decline by 0.36 fold ($p<=0.1$) and 0.28 fold ($p<=0.05$) at 3 hr and 6 hr with DD treatment. Range of molecular species was from C36 to C44 (double bond 1to 6) but none of the changes in these species were significant (data not shown). Similarly changes in another lipid class PA were not found to be significant due to its very low abundance/signal strength when compared to other lipid classes (data not shown).

To our knowledge, this is the first novel report on rapid compositional changes in certain membrane lipid classes detailed at its molecular species level in response to a PUA in algae suggesting an early regulation of molecular fatty acid species and their saturation levels that may help adapt to ROS and PUA stress in algae.

Effect of DD on SQDG lipid class in P. tricornutum

Another glycolipid class found in *P. tricornutum* called SQDG sulfoquinovosyl diacylglycerol (sulphur containing phosphorous free lipids) was unaffected by DD treatment. Overall changes in this class were not significant with DD treatment both at 3 hr and 6 hr. However lipid species such as SQDG 30:2 and 32:1 significantly increased by 1.36 ($p<0.05$) and 1.60 fold ($p<0.1$) at 3 hr with DD treatment (data not shown). With DD treatment at 6 hr, SQDG 30:1 significantly increased in by 1.2 fold ($p<0.05$) (data not shown). This lipid class is localized in thylakoid membrane and is the most saturated glycolipid. Overall, the fact that levels of SQGD are unaffected, along with other chloroplast membrane lipids such as MGDG and DGDG (Table 2.3and 2.4) supports the hypothesis that the effect of DD is on membrane lipids other than those in chloroplasts in *P. tricornutum* within 6 hr time frame.

Analysis of effect of DD on sterols in P. tricornutum

GC-MS analysis of extracted sterols from *P. tricornutum* cells showed that Brassicasterol (C-28 sterol, 24-methylcholest-5, 22-dien-3β-ol) was the dominant and major sterol that could be measured with very low amounts of campesterol (24-methylcholest-5-en-β-ol). This result agrees with earlier reports (Fabris et al., 2014; Rampen et al., 2010). Data on campesterol was not analyzed as the signal was too low in some replicates to be quantified. Peak areas of internal standard cholestenol and sterol signals were determined using chemstation software (Supplementary figure 2.14). Relative ratio/proportion of brassicasterol was measured in DMSO solvent control and DD treated cells by dividing peak area of brasiccasterol by peak area of internal standard. Fold changes in brasiccasterol in DD treated samples were calculated compared to that of DMSO solvent control.

Analysis of data (Table 2.10) shows the there was a significant ($p<0.05$) decline in brassicasterol in 10 µM DD treated cells at 3 hr to 0.86 fold. However at 6 hr with DD treatment there was no significant change in brassicasterol in DD treated cells compared to DMSO solvent control.

Sterols are also an important aspect of lipids in marine nutrition. They are essential components of the plasma membrane of all photosynthetic eukaryotes, and they control membrane fluidity and permeability (Gaulin et al., 2010; Hartman A, 1998). Along with other membrane lipids such as PC and PE, sterols help to maintain the structure and biological functionality of the membrane. Chapman (1973) reported that sterols have an effect on properties and lipid organization of membranes: they decrease fluidity, increase ordering and affect permeability of membranes. Studies done on plant sterols (sitosterol, stigmasterol and 24-methylcholesterol) have shown that sterols regulate membrane fluidity along with PC bilayers in soybean (Hartmann 1998, Schuler I et al., 1991). Studies on the water permeability of soybean phosphatidylcholine bilayers have shown that sitosterol and

24-methylcholesterol very active in reducing membrane permeability (Schuler I et al., 1991). Due to various sterols being very similar in their backbone structure it is likely that the class of sterols found in *P. tricornutum* have a similar role in maintaining membrane properties. Studies in the algae *Dunaliella* have shown that sterols play an important role in sensing osmotic changes. They propose that hyperosmotic shock induce an increase in lipid order parameters, therefore incorporation of more sterols is expected to increase the lipid order or reduce membrane fluidity (Zelazny et al., 1995). In response to DD stress, no significant change in sterols at 6 hr or an activated synthesis of sterols between 3 hr and 6 hr (after an initial decline in sterols at 3 hr) along with increases in PC and PE at 3 hr and 6 hr (Table 2.4) might indicate that cells rebuild/reorganize this essential class of lipids in the membrane to maintain the biological functions of the membrane necessary for survival. It might also help alter fluidity and permeability of the membrane to prevent further DD damage.

Marine algae are known natural source of sterols that have numerous human health benefits and applications in pharmaceuticals (Kim et al., 2011). The data showing that DD over a period of 6 hr doesn't have a major effect on sterol content in *P. tricornutum* renders its biomass still useable for commercial applications.

Effect of DD on neutral lipids

Bodipy (505/515) is a common fluorescent dye used to quantify non-polar lipids in various microalgae (Hyka P et al., 2012; Govender T et al., 2012; Cirulis J et al., 2012; Wu S, 2013). DMSO solvent controls and DD treated samples were stained with bodipy and assayed on flow cytometer to quantify non-polar lipids at 3 hr and 6 hr.

There was a 1.16 and 1.38 fold increase in non-polar lipids in 10µM DD treated cells at 3

hr and 6 hr when compared to DMSO (0.1%) solvent control (Figure 2.3). The observed response of increase in neutral lipids is similar to that observed with various other kinds of stress responses (Sharma et al., 2012) wherein cells during the time of stress instead of spending energy in expensive processes such as photosynthesis, store energy in the form of TAGs (neutral or non-polar lipids). However in most of the studies on neutral lipid accumulation (Sharma et al., 2012; Yang et al., 2014; Alipanah et al., 2015) neutral lipids were measured after days (24-72 hours or longer) indicating much higher fold accumulation. This study focused on short term lipid changes (3-6 hr) so higher levels of neutral or non-polar lipids were not expected. Our data shows that neutral lipids can start accumulating early as indicated by our fold increases in these lipids by 6hr of DD treatment. Further, observed increases in non-polar lipids may function to provide a reservoir of long chain fatty acids in rebuilding membranes necessary for survival (Solovchenko, 2012).

PUA analysis using SPME

It was suspected that observed lipid changes might lead to downstream production of volatiles or oxylipins. Analysis of volatiles using headspace SPME and GC-MS/EI of 0.1% DMSO and 10µM DD treated cells at 3 hr and 6 hr didn't show any peaks (data not shown) that might be identified as volatile compounds previously identified and described by Pohnert (2002) and Wichard (2005) from several diatom species and other algae after sonication (cell damage). This is in agreement with Wichard (2005), who reported no volatiles from 4 strains of *P. tricornutum* in their study. However control DD standard (1-10µM) dissolved in media alone (no cells) did show an expected peak, which matched that of DD. It may be that DD induced *P. tricornutum* cells don't make any volatiles or the amount made is below the sensitivity or detection limit of the technique. However Wichard (2005) couldn't detect any volatiles from sonicated *P. tricornutum* cells and Pohnert and Boland (2002) did detect non-volatiles aldehydic acids such as 9-ONDE and 12-ODTE. Further experiments are needed to understand if DD induced *P. tricornutum* cells might be making other oxylipins or above mentioned aldehydic acids from precursor fatty acids such

as 20:5 or 16:3 which might shed more light on DD induced fatty acid changes in the *P. tricornutum* cells.

Membrane permeability assays

The observed lipid changes with decadienal especially in membrane phospholipids led us to evaluate any changes in permeability of the membranes in DD treated cells. Three different types of cell permeant dyes with different molecular weights and structures were chosen to understand any altered permeability of membranes.

Evans blue dye

Evans blue dye has been extensively used in unicellular organisms including diatoms to distinguish cells with altered membrane permeability (Garrison, 2014). Our data showed that the % stained cells in DD treated samples were significantly lower (13.4 % and 13.8% at 3 hr and 6 hr respectively) compared to DMSO treated cells (21.9% and 18% at 3 hr and 6 hr) (Figure 2.4 a) indicating that somehow changes in membrane lipids due to sub-lethal 10µM DD dose treatment at 3 hr and 6 hr, affected entry of Evans blue stain into the cells. Evans blue has high molecular weight of 960.8 Da, and its 3D structure shows that it is not a planar molecule with side chains (fused rings) protruding in different planes (Supplementary figure 2.15 a). Overall complexity of structure with changed membrane properties might be a reason for low number of stained cells in DD treated samples. Figure 2.4 b shows a gray scale image taken under light microscope of a representative sample stained with Evans blue wherein gray cells indicate cells stained with Evans blue compared to non-gray cells that didn't take up the stain.

CFSE cell permeant fluorescent dye

Another dye of choice was cell permeant fluorescent stain called CFSE (Carboxyfluorescein succinimidyl ester). CFSE is a fluorescent dye, once diffuses across

membrane, is cleaved by esterase and then covalently couples, via its succinimidyl group, to intracellular molecules, particularly, to intracellular lysine residues and other amine sources) and is stable for long time once inside the cells. This dye has been widely used for studying cell division and proliferation especially lymphocyte proliferation in animal cells (Quah et al., 2007; Filby et al., 2015) and also cell divisions in algae (Hyka et al., 2013). This dye was selected for our study due to its very low toxicity, high permeability and stability once inside the cells. It was suspected that changed membrane permeability would affect the diffusion of dye across the membrane which is otherwise not an issue with normal cells. Our data showed that there was a significant decline in uptake of CFSE stain to 0.34 fold and 0.24 fold at 3 hr and 6 hr compared to DMSO solvent control, indicating altered permeability of membrane to CFSE stain in cells induced by DD (Figure 2.5a). To confirm that CFSE was localizing in the cells, fluorescence microscopy was done to observe cells under FITC filter. Figure 2.5b shows unstained cells under brightfield and FITC filter and an overlay image. There was very minimal to no background autofluorescence of the cells as can be seen in the images. Figure 2.5c shows cells stained with CFSE dye under brightfield, FITC filter (200ms exposure time), overlay image and FITC filter (800ms exposure time). The images show localization of dye inside the cells with bright fluorescent (green) signal inside the CFSE stained cells. CFSE stain has a molecular weight of 473.4 Da and its 3D structure is not planar (Supplementary figure 2.15 b), similar to Evans blue with protruding side chains in different planes which might affect its diffusion through membrane with changed lipids in response to DD. Lower fluorescence of cells may be due to reduced permeability of dye in the DD treated cells but other possibilities such as any intracellular protein changes especially in esterase enzymes and changes in the efflux mechanisms of the cells are also possible and hence needs further evaluation.

Hoechst 33342 cell permeant fluorescent stain

Hoechst is a popular cell-permeant nucleic acid fluorescent dye that diffuses across cells and binds DNA to fluoresce. It has been used as a probe to measure membrane permeability

changes in animal cells (Lalande et al., 1981; Miller et al., 1984) and more generally staining and measuring DNA content changes in animal, plants and phytoplanktons (Reynolds et al., 1996; Iwabuchi et al., 2010; Hyka et al., 2013). Our data showed that there was no significant difference in the uptake of stain between stained water control, stained DMSO control and stained DD cells at 3 hr and 6 hr (Figure 2.6a). This was different from data observed for the above two dyes namely Evans blue and CFSE. This showed that although permeability of Evans blue and CSFE dyes were affected, there was no effect of permeability of Hoechst dye in DD treated cells. Figure 2.7b shows microscope images of cells stained with Hoechst under DAPI filter (blue) showing it's binding to nuclei region in the cells. Under TRITC filter red autofluorescence of pigments was seen (Figure 2.6b, right). Hoechst dye has a molecular weight of 561.9 Da and its structure is quite planar compared to other two dyes, which might be a reason why its permeability didn't get affected. Hence these results show that DD induced lipid changes might have altered the permeability of the membranes to certain molecules but it could also be dependent on the structure and chemical nature and volume of the molecules.

Conclusions

To our knowledge this is the first study documenting changes in lipids *in Phaeodactylum tricornutum* in response to external application of DD. DD induced changes were seen mostly in membrane phospholipids without much effect on chloroplast lipids. Sterols in the membrane after an initial decline remained unaffected overall. There was a shift towards saturated and monounsaturated fatty acids compared to polyunsaturated fatty acids which might alter membrane properties such permeability, fluidity etc. for the organism's adaptive benefits. Also this shift to saturated and monounsaturated fatty acids is also desirable for application of using algae biomass for extraction of lipids for conversion to second generation biofuels. Studies on how changes in membrane lipids might be affecting its permeability showed that two out of three cellular dyes had reduced permeability into

the cells indicating that both molecular structure of the dyes and altered membrane properties might be responsible for this. Further studies are needed to understand how changes in membrane lipids affect various other membrane properties such as fluidity, ion exchange/transport etc. Further apart from determining overall permeability of the membrane, it would be interesting to determine specific permeability of DD or other oxylipin using the fluorescent tagged probes (Wolfram et al., 2014, 2015) or measuring concentration inside the cells by derivatization and mass spec analysis. Overall this study shows the DD induces rapid membrane lipid remodeling for adaptation to changed environmental conditions.

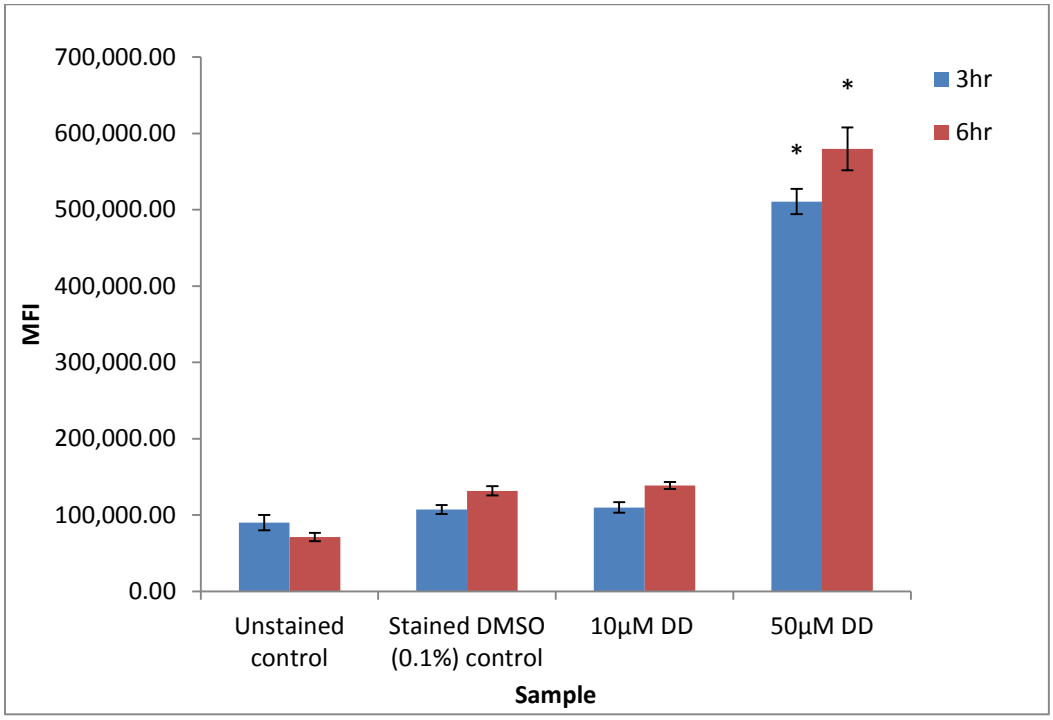


Figure 2.1: Measurement of cell viability for cells in stationary phase in response to DD dose within 6 hr using sytox green fluorescent dye by flow cytometry. Error bars represent standard error (Changes with 50µM DD were found significant with respect to DMSO solvent control, Student's T-test, * $P<0.05$; n=3).

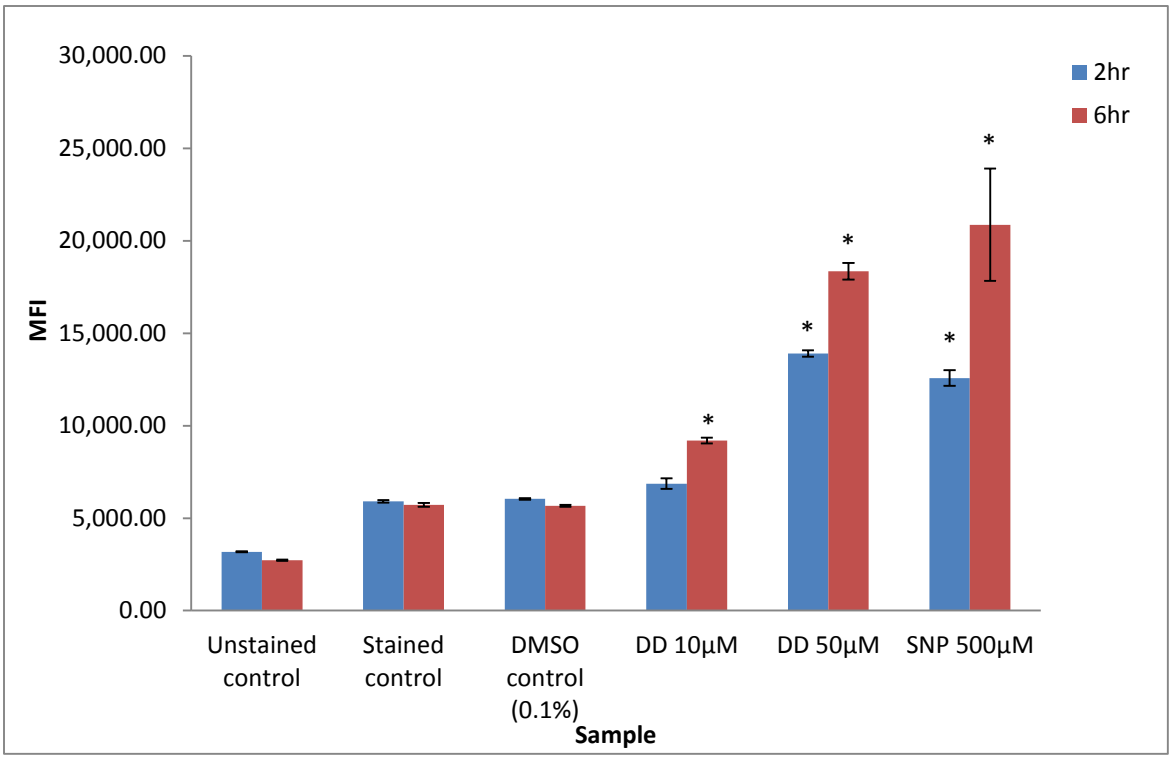


Figure 2.2: Effect of DD dose and time on NO accumulation in cells (stationary phase) measured using fluorescent dye DAF-FM diacetate by flow cytometry. Unstained control and stained DMSO (0.1%) solvent control were used as negative controls. SNP (which is a NO donor) at 500µM conc. acted as positive control. Error bars represent standard error. (*Significance calculated by student's t-test, $P<0.05$, n=3)).

Table 2.1: Fold changes in measured saturated and unsaturated fatty acids in 10μM DD treated cells compared to DMSO solvent control at 3 hr and 6 hr.

	Fold change in fatty acids in DD treated cells compared to DMSO solvent control	
	3 hr	**6 hr**
Saturated FAs		
14:0	0.68** (0.02)	0.81**(0.12)
16:0	0.69* (0.18)	0.92 (0.09)
18:0	0.57** (0.15)	0.74**(0.11)
Unsaturated FAs		
16:1	0.62* (0.22)	0.87 (0.12)
16:2	0.48** (0.16)	0.84** (0.10)
16:2	0.52** (0.16)	0.73** (0.08)
16:2	0.52** (0.19)	0.82 (0.17)
16:3	0.57** (0.14)	0.80** (0.08)
18:1	0.86 (0.19)	1.12** (0.06)
18:2	0.66* (0.20)	1.46** (0.16)
20:5	0.56* (0.24)	0.82** (0.07)
22:6	0.52** (0.16)	0.81** (0.10)

(Data presented for 3h time point is representative of two independent experiments; 5-8 biological replicates each for DMSO and DD treatments. Data for 6 hr time point is representative of three independent experiments; 5-8 biological reps each for DMSO and DD treatments; numbers in brackets indicate ±SD; Significance * P<=0.1 and ** P<0.05 calculated by One sample student's t-test).

Table 2.2: Analysis of changes in total saturated and monounsaturated fatty acids compared to that of total polyunsaturated fatty acids in DD treated cells at 3 hr and 6 hr.

	Ratio at 3 hr (FA Peak Area / Internal Standard Peak Area)		Ratio at 6 hr (FA Peak Area / Internal Standard Peak Area)		Fold Change in DD treated cells compared to DMSO solvent control	
	DMSO (0.1%)	10µM DD	DMSO (0.1%)	10µM DD	3 hr	6 hr
Total SFAs+MUFAs	1.46 (0.05)	0.96 (0.24)	1.38 (0.94)	1.29 (0.20)	0.65*	0.94
Total PUFAs	1.62 (0.03)	0.89 (0.32)	1.30 (0.87)	1.13 (0.33)	0.55*	0.87

(Data presented for 3h time point is representative of two independent experiments; 5-8 biological replicates each for DMSO and DD treatments. Data for 6 hr time point is representative of three independent experiments; 5-8 biological reps each for DMSO and DD treatments; numbers in brackets indicate ±SD; Significance * P<=0.1 and ** P<0.05 calculated by Student's t-test).

Table 2.3: Mol % of lipid in each head group class in DMSO solvent and 10μM DD treated cells at 3 hr and 6 hr.

Lipid class	Lipid Mol%			
	DMSO (0.1%) control at 3 hr	10μM DD at 3 hr	DMSO (0.1%) control at 6 hr	10μM DD at 6 hr
DGDG	13.973 (0.972)	13.640 (0.779)	12.764 (1.000)	13.444 (0.360)
MGDG	43.957 (3.731)	43.139 (3.039)	41.981 (5.410)	40.695 (1.121)
LPC	11.529 (1.301)	12.362 (0.944)	12.932 (4.345)	10.707 (0.644)
PC	21.828 (1.708)	22.821 (2.066)	21.553 (1.018)	26.008** (0.834)
PG	3.715 (0.145)	2.557** (0.207)	4.402 (1.009)	4.129 (0.618)
PI	3.774 (0.306)	4.011 (0.333)	4.556 (0.888)	3.566* (0.277)
LPG	0.850 (0.156)	0.930 (0.449)	1.494 (0.643)	0.839* (0.162)
LPE	0.106 (0.040)	0.131 (0.023)	0.091 (0.053)	0.120 (0.033)
PE	0.211 (0.084)	0.377** (0.043)	0.174 (0.072)	0.471* (0.288)
PS	0.048 (0.030)	0.017* (0.006)	0.037 (0.021)	0.011** (0.005)
PA	0.009 (0.005)	0.014 (0.006)	0.014 (0.012)	0.011 (0.005)
SQDG	2.587 (0.465)	3.150 (0.510)	2.491 (0.430)	2.464 (0.262)

(Data represents average of 5 biological replicates for DMSO and DD treated cells and numbers in bracket represent standard deviations; ** $P<0.05$, * $P<0.1$ as determined by student's t-test)

Table 2.4: Fold changes in lipid classes in DD treated *P tricornutum* cells compared to DMSO solvent control

Lipid class	Fold change in DD treated cells compared to DMSO solvent control	
	3 hr	6 hr
DGDG	0.98	1.05
MGDG	0.98	0.97
LPC	1.07	0.83
PC	1.05	1.21**
PG	0.69**	0.94
PI	1.06	0.78*
LPG	1.09	0.56*
LPE	1.24	1.32
PE	1.79**	2.70*
PS	0.36*	0.28**
PA	1.58	0.77

(Data represents fold changes calculated from 5 bio reps of each DMSO and DD treated cultures; ** $p<0.05$; * $p<0.1$ determined by student's t-test based on Table 2.1)

Table 2.5: Changes in mol % of lipid molecular species of PE lipid class in DMSO solvent (0.1%) control and 10µM DD treated cells.

	PE lipid class			
Lipid Molecular species	**Mol% at 3 hr**		**Mol% at 6 hr**	
	DMSO (0.1%)	**10µM DD**	**DMSO (0.1%)**	**10µM DD**
32:1	0.005 (0.002)	0.009 (0.001)	0.004 (0.002)	0.013 (0.006)
32:0	0.000 (0.000)	0.000 (0.000)	0.001 (0.001)	0.000 (0.001)
34:1	0.001 (0.001)	0.004 (0.001)	0.002 (0.003)	0.006 (0.004)
36:1	0.000 (0.000)	0.003 (0.001)	0.000 (0.000)	0.003 (0.003)
Total SFA+MUFAs	**0.007**	**0.016**	**0.007**	**0.023**
32:3	0.001 (0.001)	0.001 (0.001)	0.001 (0.001)	0.002 (0.001)
32:2	0.007 (0.003)	0.012 (0.003)	0.005 (0.002)	0.010 (0.008)
34:4	0.002 (0.002)	0.004 (0.001)	0.002 (0.001)	0.005 (0.004)
34:3	0.008 (0.003)	0.012 (0.001)	0.005 (0.003)	0.012 (0.009)
34:2	0.007 (0.004)	0.012 (0.003)	0.007 (0.002)	0.017 (0.010)
36:6	0.017 (0.008)	0.031 (0.004)	0.011 (0.007)	0.032 (0.020)
36:5	0.010 (0.005)	0.019 (0.001)	0.010 (0.002)	0.022 (0.012)
36:4	0.003 (0.002)	0.005 (0.002)	0.003 (0.002)	0.005 (0.003)
36:3	0.001 (0.001)	0.002 (0.002)	0.002 (0.001)	0.001 (0.001)
36:2	0.004 (0.002)	0.022 (0.006)	0.006 (0.003)	0.030 (0.017)
38:8	0.008 (0.003)	0.013 (0.003)	0.005 (0.004)	0.016 (0.009)
38:7	0.030 (0.010)	0.047 (0.006)	0.021 (0.009)	0.060 (0.035)
38:6	0.009 (0.004)	0.025 (0.003)	0.004 (0.004)	0.037 (0.022)
38:5	0.001 (0.001)	0.002 (0.001)	0.001 (0.001)	0.001 (0.001)
40:10	0.039 (0.015)	0.053 (0.005)	0.032 (0.015)	0.072 (0.049)
40:9	0.010 (0.005)	0.016 (0.003)	0.009 (0.006)	0.016 (0.010)
40:8	0.005 (0.002)	0.006 (0.003)	0.004 (0.002)	0.008 (0.006)
42:11	0.042 (0.019)	0.078 (0.008)	0.039 (0.015)	0.102 (0.064)
PUFAs total	**0.204**	**0.360**	**0.167**	**0.448**

(Data is average of 5 biological replicates and values in bracket represent standard deviation; for information on statistical significance, see text).

Table 2.6: Changes in mol % of lipid molecular species of PC lipid class in DMSO solvent (0.1%) control and 10μM DD treated cells.

	PC lipid class			
Lipid Molecular species	**Mol% at 3 hr**		**Mol% at 6 hr**	
	DMSO (0.1%)	**10μM DD**	**DMSO (0.1%)**	**10μM DD**
30:1	0.190 (0.033)	0.182 (0.012)	0.200 (0.019)	0.194 (0.009)
30:0	0.233 (0.028)	0.227 (0.026)	0.253 (0.037)	0.249 (0.014)
32:1	1.037 (0.109)	1.221 (0.106)	1.019 (0.055)	1.305 (0.049)
32:0	0.187 (0.024)	0.288 (0.045)	0.219 (0.079)	0.266 (0.021)
34:1	0.260 (0.050)	0.833 (0.076)	0.287 (0.078)	1.067 (0.102)
36:1	0.064 (0.005)	0.100 (0.014)	0.049 (0.021)	0.102 (0.024)
Total SFA+MUFAs	**1.972**	**2.851**	**2.026**	**3.183**
32:4	0.129 (0.036)	0.127 (0.023)	0.122 (0.012)	0.101 (0.009)
32:3	0.190 (0.040)	0.208 (0.032)	0.186 (0.017)	0.184 (0.012)
32:2	0.551 (0.045)	0.551 (0.053)	0.544 (0.053)	0.563 (0.035)
34:5	0.521 (0.039)	0.441 (0.041)	0.589 (0.078)	0.452 (0.036)
34:4	0.334 (0.042)	0.254 (0.021)	0.349 (0.052)	0.250 (0.006)
34:3	0.839 (0.049)	0.722 (0.086)	0.840 (0.064)	0.863 (0.049)
34:2	1.044 (0.099)	1.292 (0.121)	1.124 (0.157)	1.688 (0.089)
36:8	0.219 (0.066)	0.229 (0.042)	0.171 (0.023)	0.195 (0.011)
36:7	0.222 (0.042)	0.168 (0.030)	0.204 (0.036)	0.155 (0.017)
36:6	1.335 (0.090)	1.314 (0.132)	1.290 (0.098)	1.239 (0.125)
36:5	3.266 (0.354)	3.198 (0.415)	3.201 (0.151)	3.755 (0.212)
36:4	0.265 (0.044)	0.373 (0.068)	0.248 (0.037)	0.249 (0.048)
36:3	0.257 (0.031)	0.305 (0.043)	0.253 (0.064)	0.339 (0.022)
36:2	0.306 (0.023)	0.318 (0.038)	0.380 (0.118)	0.363 (0.030)
38:9	0.490 (0.025)	0.272 (0.034)	0.594 (0.120)	0.262 (0.013)
38:8	0.573 (0.075)	0.378 (0.050)	0.593 (0.134)	0.517 (0.052)
38:7	2.610 (0.142)	2.307 (0.237)	2.286 (0.169)	2.673 (0.145)
38:6	0.915 (0.187)	2.119 (0.173)	0.862 (0.171)	3.558 (0.478)
38:5	0.212 (0.025)	0.208 (0.017)	0.191 (0.052)	0.053 (0.080)
38:4	0.043 (0.026)	0.083 (0.016)	0.047 (0.023)	0.116 (0.016)
38:3	0.048 (0.007)	0.054 (0.015)	0.044 (0.009)	0.052 (0.005)

Table 2.6 continued…

38:2	0.064	(0.007)	0.068	(0.010)	0.054	(0.019)	0.067	(0.012)
40:10	2.584	(0.249)	2.212	(0.229)	2.647	(0.305)	2.332	(0.226)
40:9	0.713	(0.122)	0.463	(0.042)	0.637	(0.093)	0.394	(0.038)
40:8	0.471	(0.030)	0.501	(0.043)	0.407	(0.031)	0.530	(0.030)
40:7	0.143	(0.028)	0.208	(0.027)	0.108	(0.019)	0.181	(0.022)
40:5	0.048	(0.010)	0.059	(0.007)	0.045	(0.016)	0.049	(0.007)
40:4	0.031	(0.002)	0.041	(0.007)	0.044	(0.014)	0.039	(0.013)
40:3	0.053	(0.005)	0.059	(0.012)	0.051	(0.018)	0.061	(0.006)
40:2	0.114	(0.011)	0.119	(0.010)	0.114	(0.034)	0.133	(0.006)
42:11	0.916	(0.064)	0.966	(0.104)	0.943	(0.045)	1.065	(0.052)
42:10	0.081	(0.011)	0.074	(0.014)	0.064	(0.015)	0.054	(0.031)
42:3	0.087	(0.003)	0.103	(0.013)	0.085	(0.025)	0.093	(0.004)
42:2	0.089	(0.016)	0.077	(0.020)	0.115	(0.060)	0.106	(0.016)
44:12	0.052	(0.008)	0.063	(0.007)	0.052	(0.014)	0.060	(0.006)
44:6	0.039	(0.008)	0.038	(0.006)	0.043	(0.009)	0.036	(0.006)
PUFAs total	**19.857**		**19.970**		**19.527**		**22.825**	

(Data is average of 5 biological replicates and values in bracket represent standard deviation; for information on statistical significance, see text).

Table 2.7: Changes in mol% of lipid molecular species of LPE lipid class in DMSO solvent (0.1%) control and 10μM DD treated cells.

	LPE lipid class			
Lipid Molecular species	**Mol% at 3 hr**		**Mol% at 6 hr**	
	DMSO (0.1%)	**10μM DD**	**DMSO (0.1%)**	**10μM DD**
16:1	0.007 (0.005)	0.016 (0.009)	0.006 (0.005)	0.013 (0.012)
16:0	0.015 (0.007)	0.011 (0.010)	0.018 (0.015)	0.012 (0.006)
18:1	0.011 (0.008)	0.022 (0.016)	0.011 (0.008)	0.037 (0.009)
Total SFA+MUFAs	**0.033**	**0.049**	**0.035**	**0.062**
18:3	0.007 (0.003)	0.010 (0.006)	0.005 (0.004)	0.003 (0.003)
18:2	0.040 (0.022)	0.048 (0.007)	0.029 (0.014)	0.035 (0.007)
22:6	0.025 (0.007)	0.024 (0.001)	0.021 (0.014)	0.020 (0.011)
Total PUFAs	**0.073**	**0.082**	**0.055**	**0.058**

(Data is average of 5 biological replicates and values in bracket represent standard deviation; for information on statistical significance, see text).

Table 2.8: Changes in mol % of lipid molecular species of LPC lipid class in DMSO solvent (0.1%) control and 10µM DD treated cells.

	LPC lipid class			
Lipid Molecular species	**Mol% at 3 hr**		**Mol% at 6 hr**	
	DMSO (0.1%)	10µM DD	DMSO (0.1%)	10µM DD
16:1	0.949 (0.123)	0.968 (0.070)	1.003 (0.197)	0.773 (0.054)
16:0	2.347 (0.206)	2.569 (0.255)	2.789 (1.271)	1.898 (0.108)
18:1	1.076 (0.430)	2.645 (0.188)	1.378 (0.784)	2.713 (0.249)
18:0	0.097 (0.006)	0.099 (0.021)	0.122 (0.056)	0.047 (0.015)
Total SFA+MUFAs	**4.469**	**6.281**	**5.292**	**5.431**
18:3	0.532 (0.055)	0.398 (0.039)	0.646 (0.175)	0.356 (0.033)
18:2	2.591 (0.230)	2.228 (0.163)	2.368 (0.655)	2.000 (0.185)
20:5	2.900 (0.335)	2.362 (0.277)	3.231 (0.660)	2.018 (0.111)
22:6	1.037 (0.078)	1.092 (0.098)	1.396 (0.799)	0.902 (0.076)
Total PUFAs	**7.060**	**6.080**	**7.641**	**5.276**

(Data is average of 5 biological replicates and values in bracket represent standard deviations; for information on statistical significance, see text).

Table 2.9: Summary of fold changes in saturated and monounsaturated fatty acids (SFAs and MUFAs) and polyunsaturated fatty acids (PUFAs) in PE, PC, LPE and LPC lipid classes in DD treated cells at 3 hr and 6 hr.

	Fold changes in DD treated cells compared to DMSO solvent control							
	PE		**PC**		**LPC**		**LPE**	
	3 hr	**6 hr**	**3 hr**	**6 hr**	**3 hr**	**6 hr**	**3 hr**	**6 hr**
SFAs+MUFAs	**2.33**	**3.35**	**1.45**	**1.57**	**1.41**	**1.03**	**1.47**	**1.76**
PUFAs	**1.77**	**2.68**	**1.01**	**1.17**	**0.86**	**0.69**	**1.13**	**1.04**

Table 2.10: Average relative ratio of brassicasterol in DMSO solvent and DD treated samples and fold changes in brassicasterol in 10µM DD treated cells compared to DMSO solvent control at 3 hr and 6 hr.

	Ratio at 3 hr		**Ratio at 6 hr**		**Fold change in DD treated cells compared to DMSO solvent control at 3 hr**	**Fold change in DD treated cells compared to DMSO solvent control at 6 hr**
	DMSO (0.1%) solvent control	**DD 10µM**	**DMSO (0.1%) solvent control**	**DD 10µM**		
Brassicasterol (C-28 sterol)	1.52 (0.03)	1.30 (0.08)	1.55 (0.11)	1.46 (0.14)	0.86**	0.95

(Data represents average of 5 biological replicates for DD treatment and 4 biological replicates for DMSO solvent, values in bracket represents SD; **$p<0.05$ determined by student's t-test)

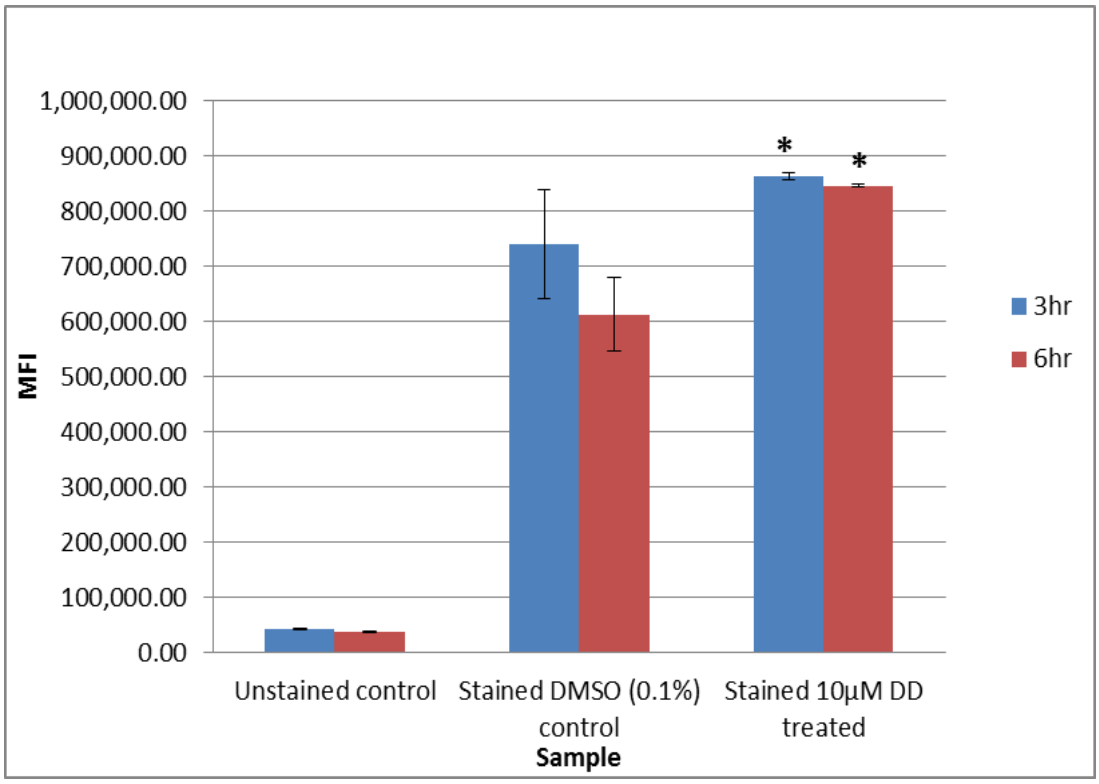
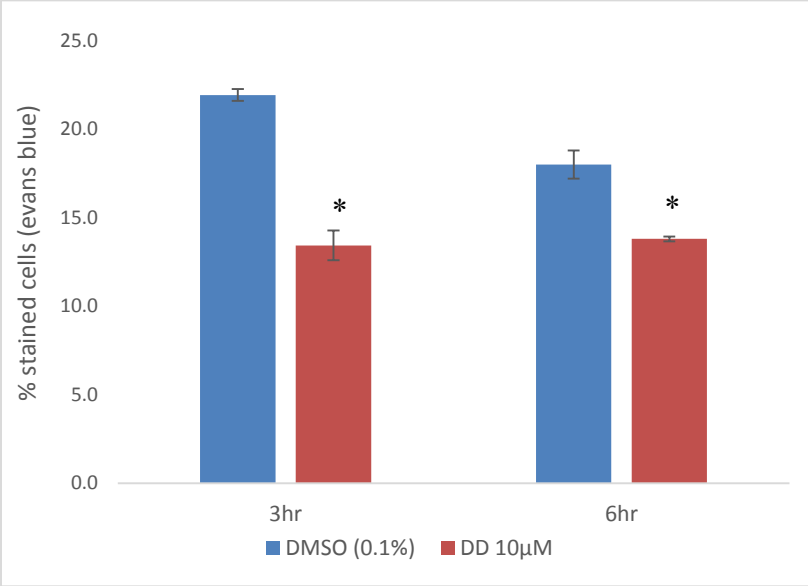


Figure 2.3: Non-polar lipids as quantified by Bodipy dye (505/515) on flow cytometer. Error bars represent standard error between three biological replicates (* $P<0.05$ with respect to DMSO solvent control). This data is representative of three experiments.

a)



b)

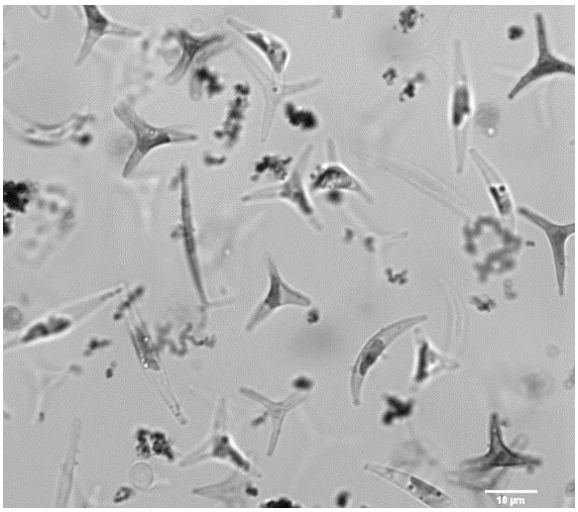


Figure 2.4: a) Analysis of % stained cells by cell counting on hemocytometer b) Bright field image (630X) of cells stained with evans blue dye (scale bar= 10µM). Data representative of 3 independent experiments; 3 replicates, $p<0.05$)

a)

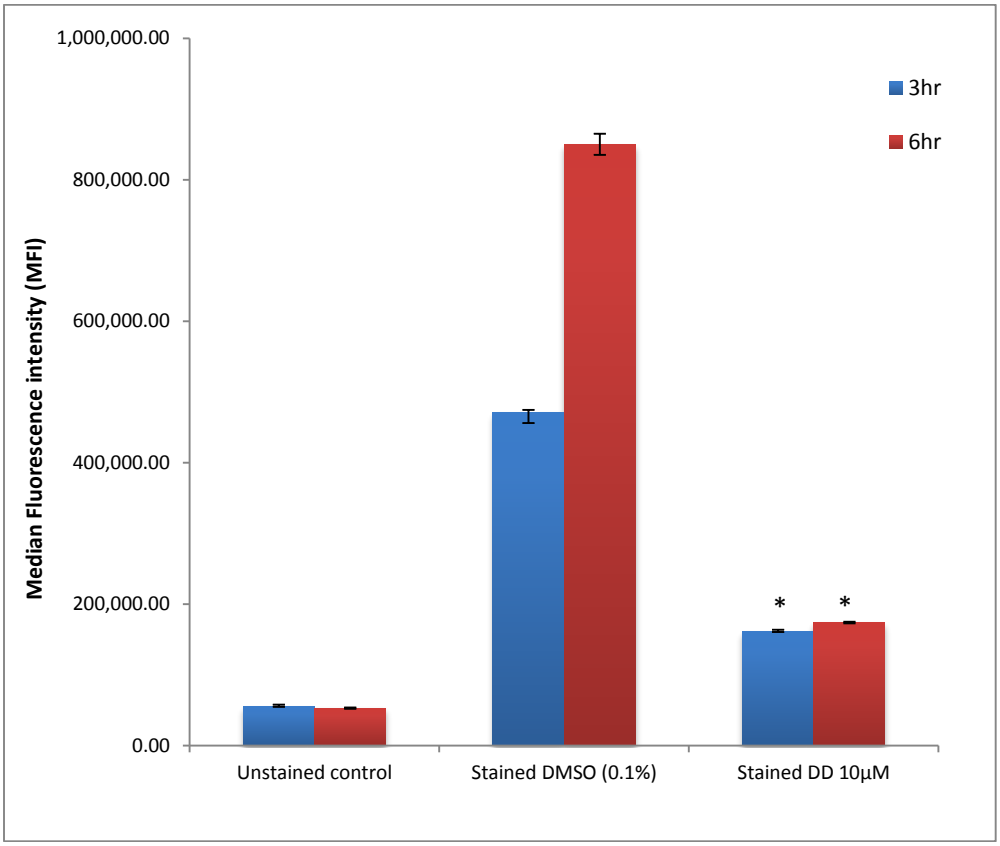
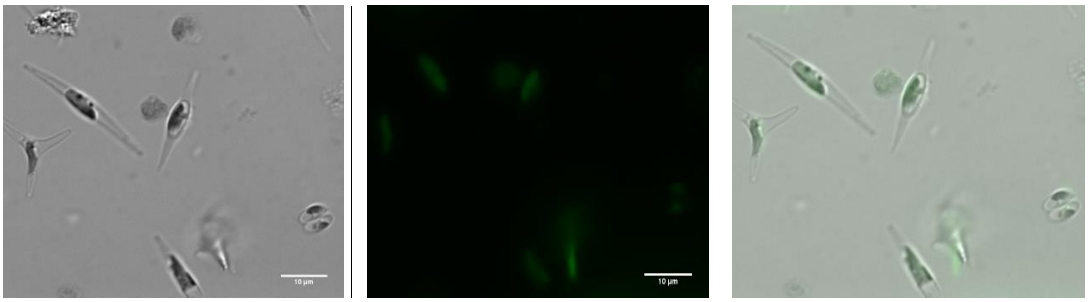


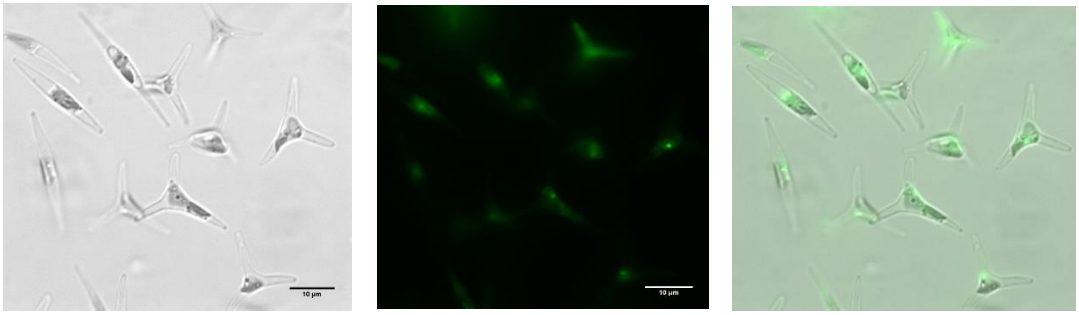
Figure 2.5 continued next page…

b)

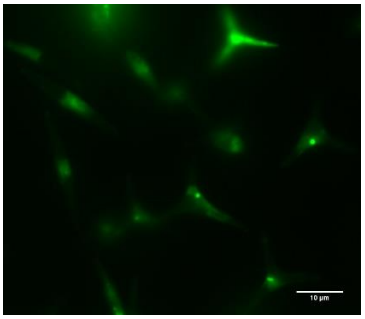


Unstained (BF) (FITC-exposure 1sec) overlay

c)



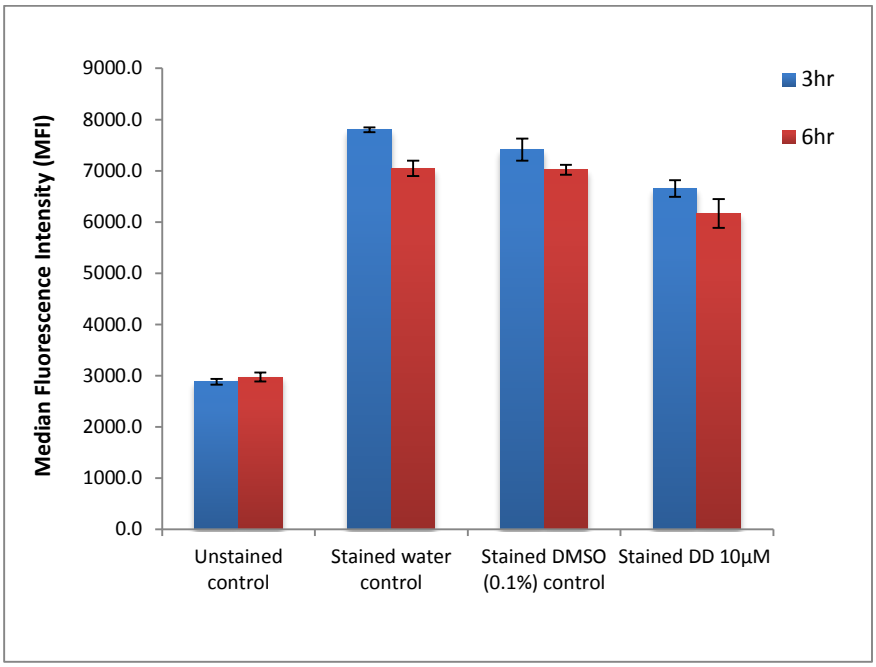
Stained (BF) FITC (exposure 200msec) overlay



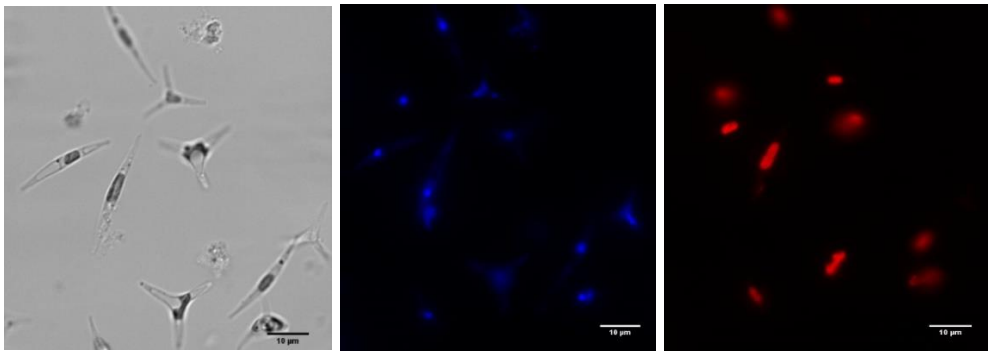
FITC (exposure 800msec)

Figure 2.5: a) Analysis of fluorescence of stained cells by flow cytometer (BD Acurri) using CFSE stain b) Bright field image and fluorescence image under FITC (green) of unstained cells and c) cells stained with CFSE dye at 630X magnification (data representative of 3 independent experiments; 3 replicates, $p<0.05$)

a)

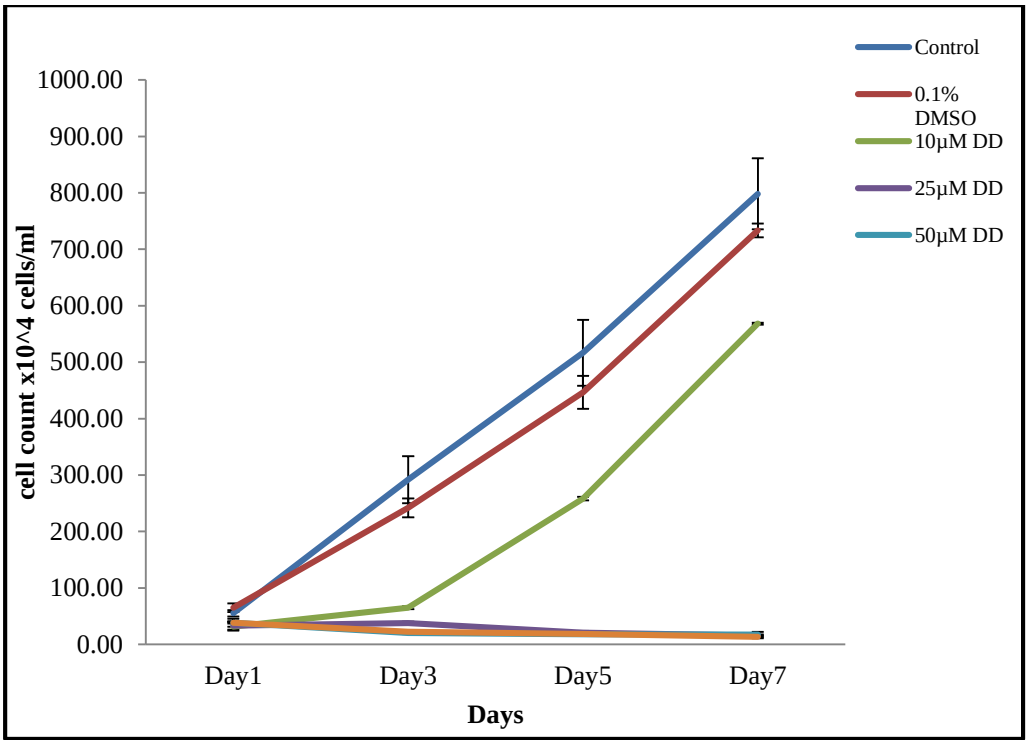


b)

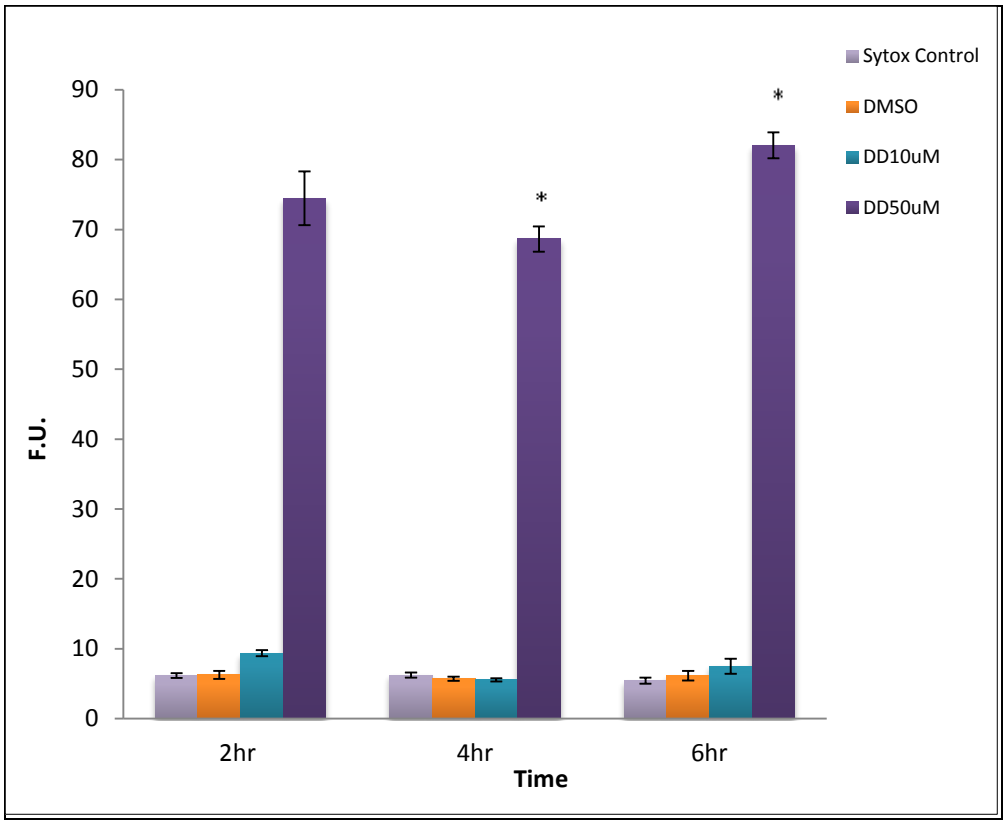


Brightfield DAPI filter TRITC filter (autofluorescence of cells)

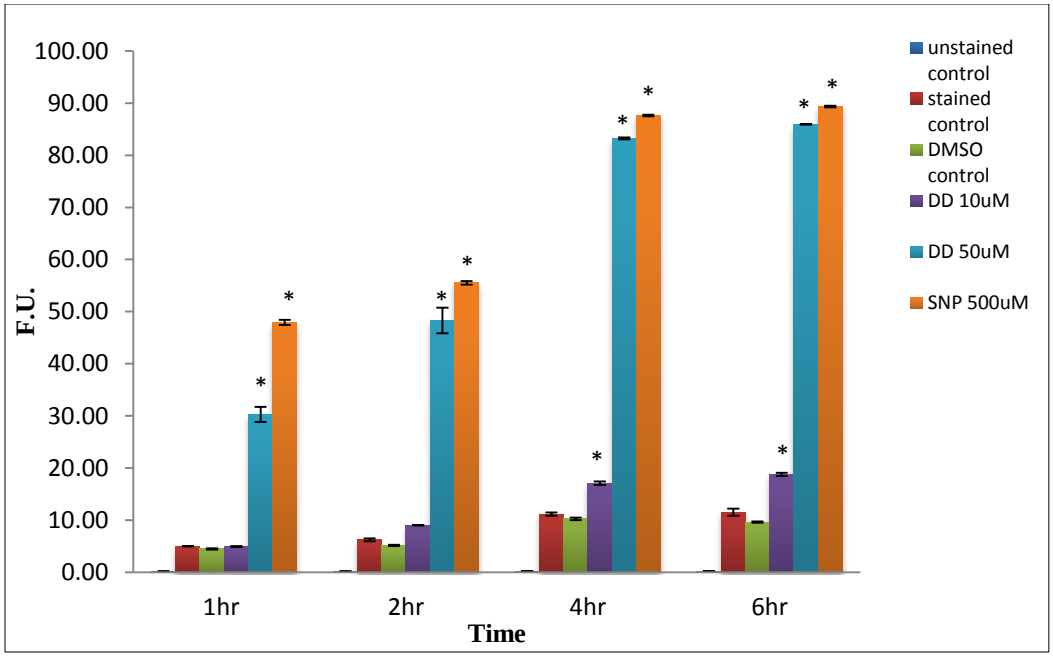
Figure 2.6: a) Analysis of fluorescence of stained cells by flow cytometer (BDFACS Aria) using Hoechst 33342 stain b) Bright field image, fluorescence image under DAPI (blue) of stained cells; Autofluorescence of cells (Red) under TRITC filter at 630X magnification (Data representative of 3 independent experiments; 3 replicates, $p<0.05$)



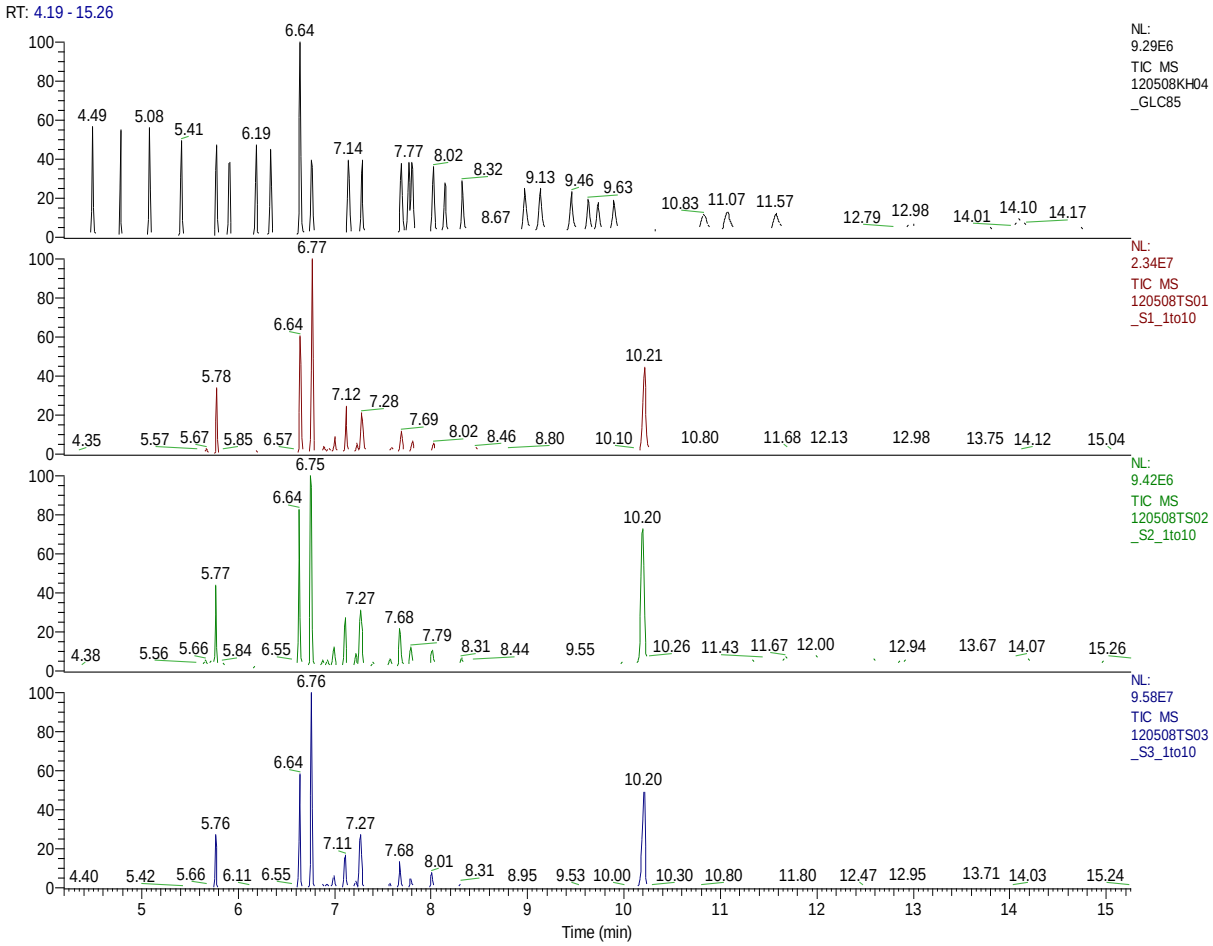
Supplementary Figure 2.7: Effect of DD dose on cell growth measured for 7 days by doing cell counts using hemocytometer. Error bars represent standard error. ($p < 0.05$ for 25µM, 50µM and 75µM DD with respect to DMSO solvent control and $p > 0.05$ for 10µM DD with respect to DMSO solvent control; n=3).Representative data from three independent experiments is shown here.



Supplementary Figure 2.8: Measurement of cell viability for cells in exponential phase in response to DD dose within 6 hr using sytox green fluorescent dye by flow cytometry. Error bars represent standard error (Changes with 50µM DD were found significant with respect to DMSO solvent control, Student's T-test, * $p<0.05$; n=3).

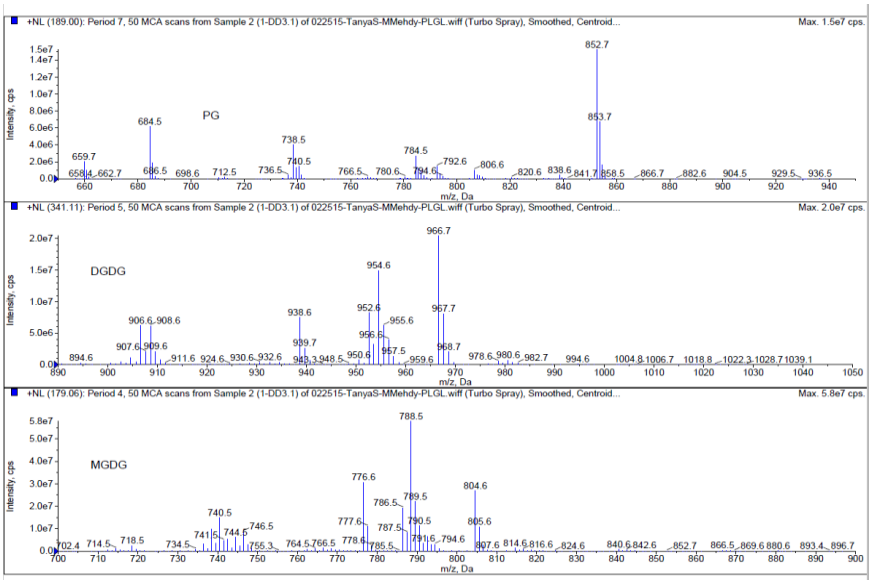


Supplementary Figure 2.9: Effect of DD dose and time on NO accumulation in cells (exponential phase) measured using fluorescent dye DAF-FM diacetate by flow cytometry. Unstained and stained water controls and DMSO (0.1%) solvent control were used as negative controls. SNP (which is a NO donor) at 500µM conc. acted as positive control. Error bars represent standard error. (*Significance calculated by student's t-test, $p<0.05$, n=3)). This data is representative of 3 independent experiments.

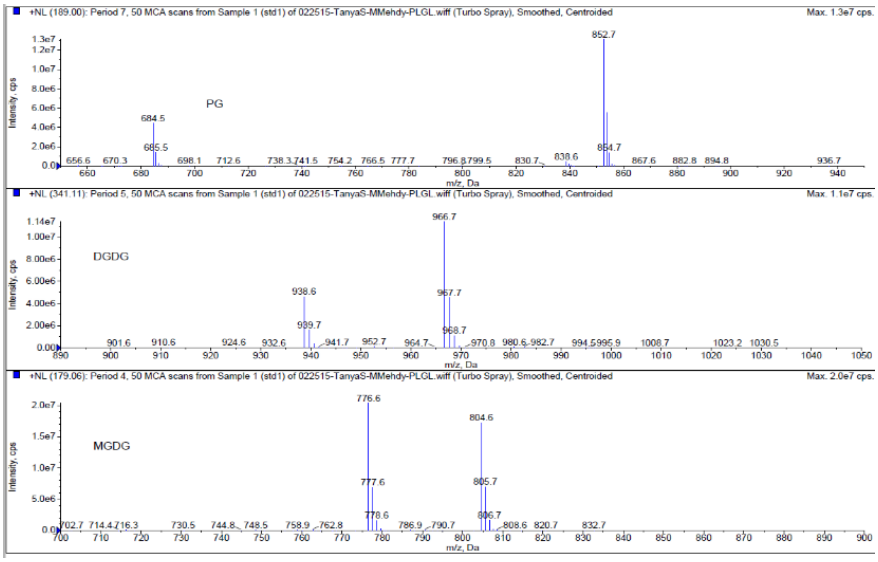


Supplementary Figure 2.10: Representative GC chromatogram of FAME standard mix (GLC85) (first chromatogram) and FAMEs from *P. tricornutum* samples (Bottom three chromatograms). Identified fatty acid peaks at retention times (RT) are as follows: RT 5.77=14:0; RT 6.64= 16:0, RT 6.77= 16:1; RT 6.84-7.02= 16:2; RT 7.12= 16:3; RT 7.68-69= 18:0; RT 7.81-84= 18:1; RT 8.01= 18:2; RT 10.20= 20:5; RT 12.98-13.01= 22:6.

a)

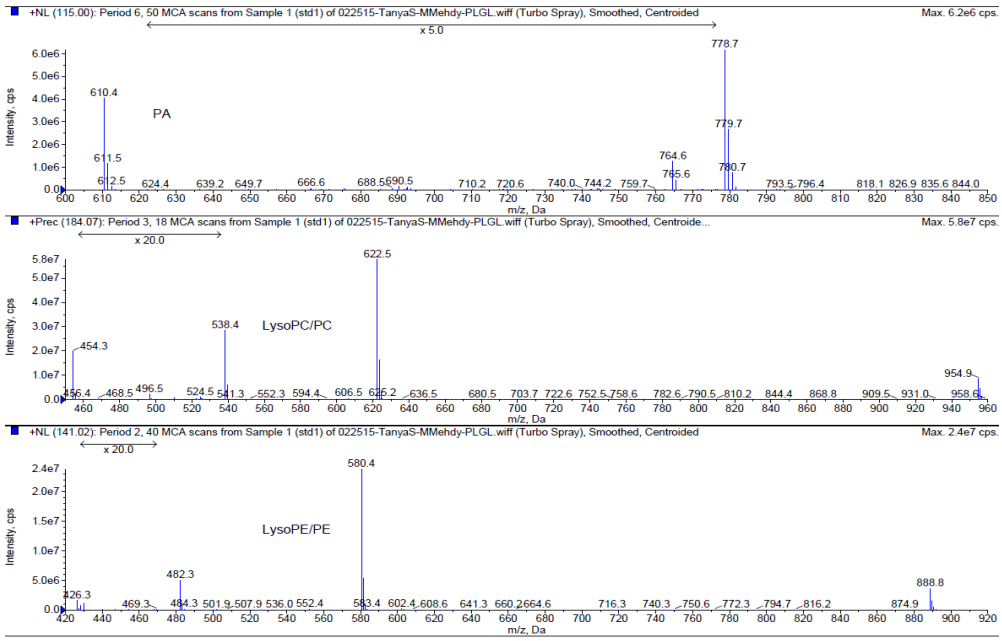


b)

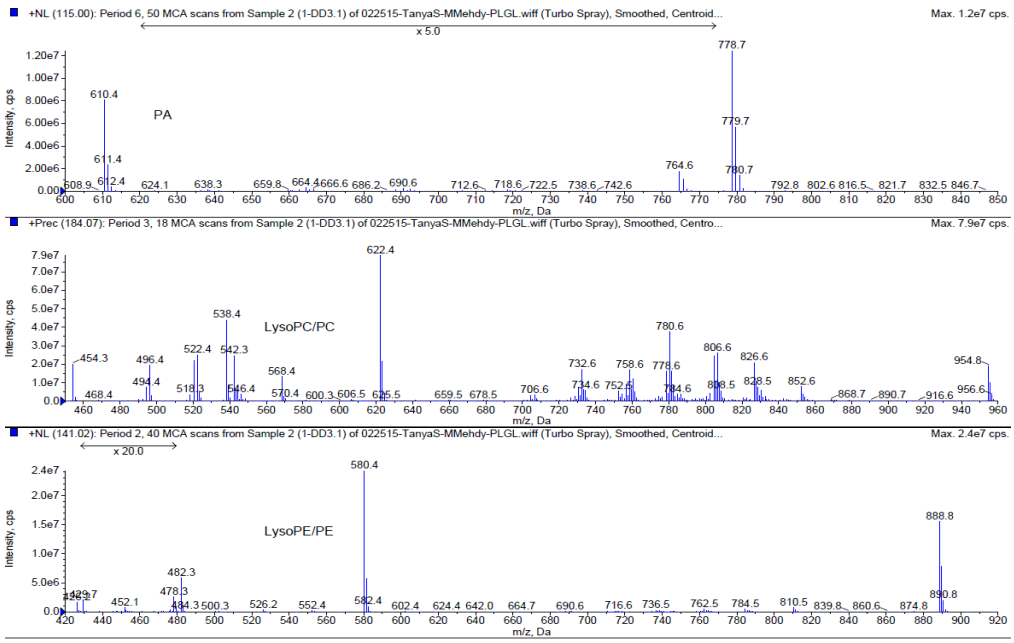


Supplementary Figure 2.11: Representative ESI-MS Spectra of lipid classes PG, MGDG and DGDG a) in standards b) in *P. tricornutum* sample

a)

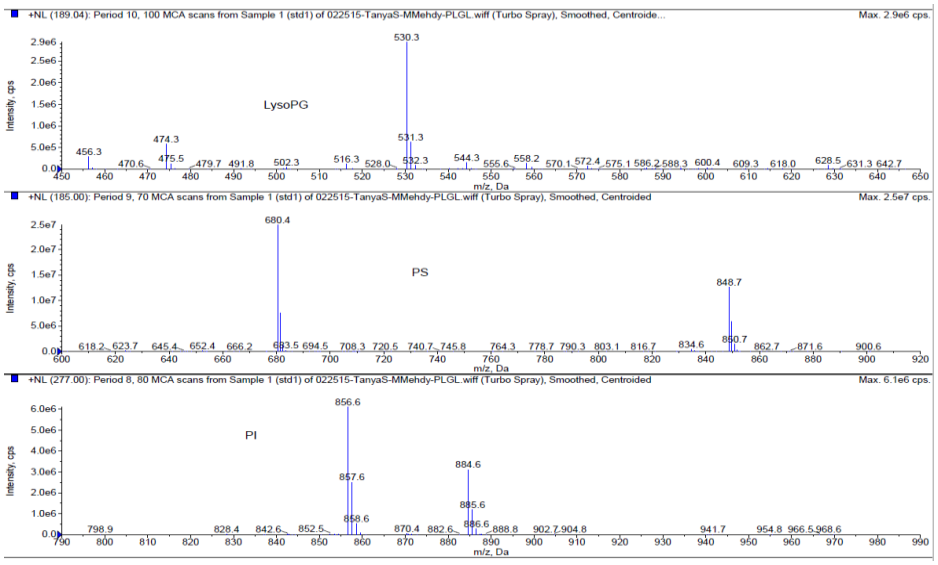


b)

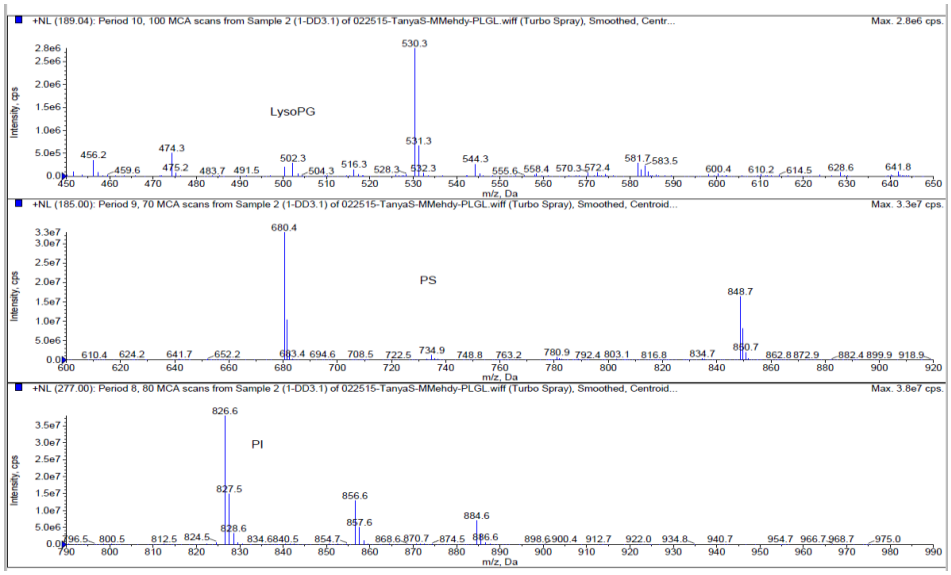


Supplementary Figure 2.12: Representative ESI-MS Spectra of lipid classes PA, Lyso PC/PC and LysoPE/PE a) in standards b) in *P. tricornutum* sample.

a)

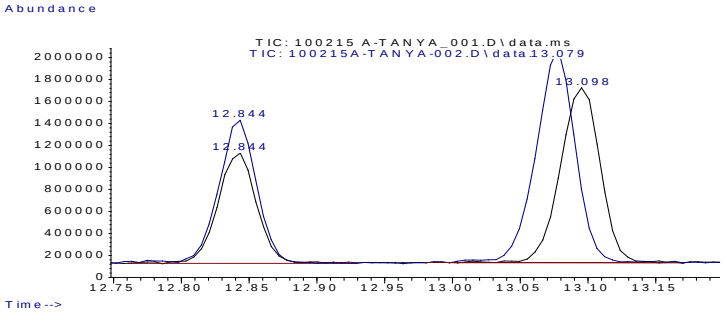


b)

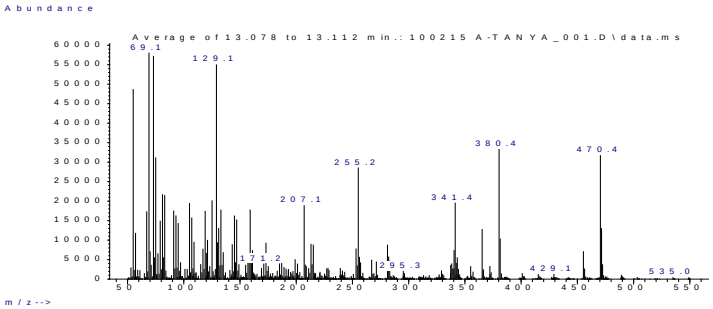


Supplementary Figure 2.13: Representative ESI-MS Spectra of lipid classes Lyso PG, PS and PI a) in standards b) in *P. tricornutum* sample.

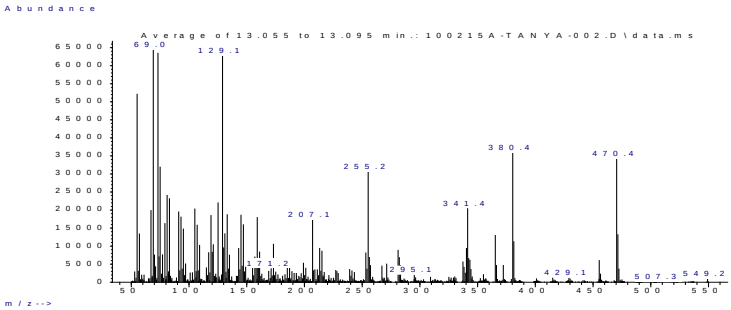
a)



b)

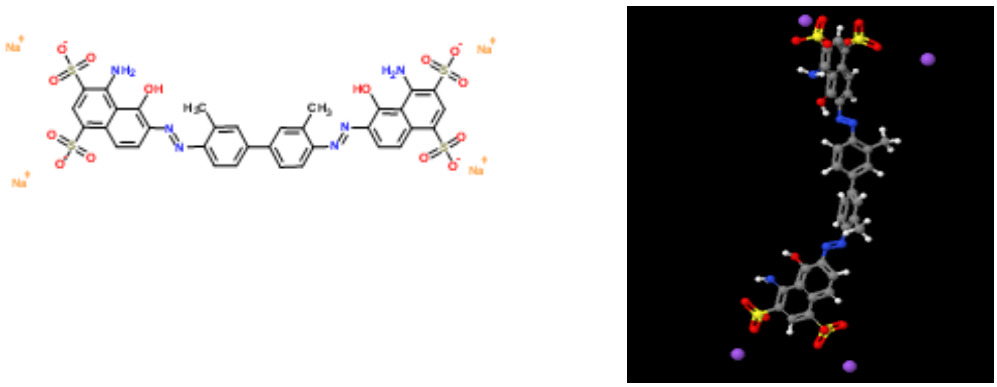


c)

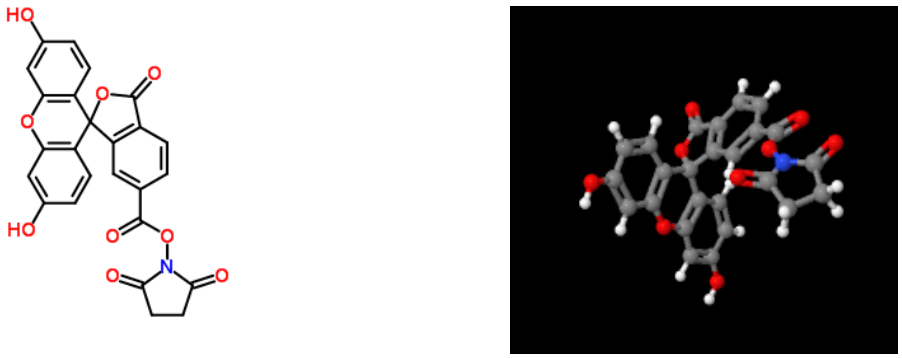


Supplementary Figure 2.14: Representative a) Overlaid Chromatogram of sterol (brassicasterol) standard (black) and that in P tricornutum sample (blue); RT 12.84 = Internal standard Cholestanol; RT 13.07-13.09= Brassicasterol and b) MS Spectra of standard of sterol (brassicasterol) b) MS Spectra of brassicasterol in *P. tricornutum* sample.

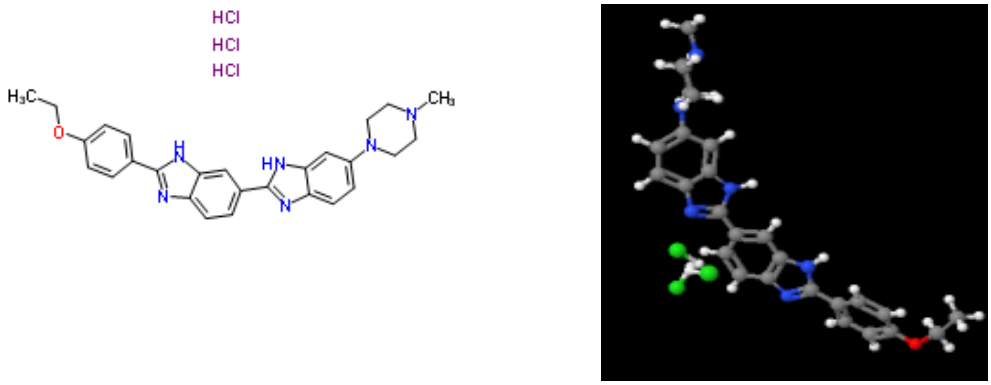
a)



b)



c)



Supplementary Figure 2.15: Structures of dyes a) Evans Blue b) CFSE (Carboxyfluorescein succinimidyl ester) c) Hoechst 33342

Chapter 3: Decadienal induced transcriptome regulation with focus on lipid metabolism processes in the diatom *Phaeodactylum tricornutum*

Abstract

Diatoms are an ecologically important and successful group of unicellular algae that have developed mechanisms to survive and adapt to highly variable habitats, confronting a variety of abiotic and biotic stresses. Many diatoms release oxylipins (including PUAs and oxygenated lipids) as a defense mechanism that inhibit reproduction in grazers and signals undamaged diatom cells. One such model PUA is decadienal that has been studied for its effect on both grazers and diatoms. In this study, we analyzed global transcriptome changes with focus on lipid metabolism pathways in the marine diatom *Phaeodactylum tricornutum* in response to sub-lethal dose of decadienal within a short time scale (3 hr, 6 hr) by RNA Sequencing. This study was done with an attempt to correlate transcriptome profiling to our earlier study on changes in fatty acids and lipid classes in response to decadienal. Out of 12321 predicted mapped transcripts (genes), 7320 and 4631 genes were differentially regulated ($q<0.05$) in response to decadienal at 3 hr and 6 hr, respectively. Analysis of Gene Ontology (GO) groups enriched in upregulated genes were primarily those involved in lipid metabolism, RNA metabolism, RNA processing, protein catabolism, translation and amino acid synthesis, carbohydrate and organic acid metabolic processes. Whereas GO groups that were downregulated were those involved in photosynthesis, generation of precursor metabolites and energy, DNA metabolic processes, various ion transport processes and chromosome/chromatin organization. Among highly upregulated genes were those involved in regulation of oxidative stress and defense or detoxification mechanisms. Analysis of genes in lipid metabolic GO group showed downregulation of genes that initiate fatty acid synthesis (14:0, 16:0 and 18:0 fatty acids) in chloroplast. This correlates to our earlier data on overall lower measured lipid levels in response to DD. However several genes (PTD6, FAD2 (Δ9 and Δ12 desaturases), PTD9, PTD5a) involved in synthesis of unsaturated fatty acids like 18:1, 18:2, 20:5 in cytosol were upregulated.

This agrees with earlier observed increased levels of 18:1 and 18:2 fatty acids however we reported decline in levels of 20:5. Also genes involved in synthesis of TAGs were downregulated and genes such as lipases and oxidases involved in lipid degradation were also downregulated which supports our earlier detected minimal increases in TAG levels whereas increases in some membrane phospholipids were more prominent. These results indicate restructuring of carbon metabolism and coordination of multiple metabolic pathways at transcriptome levels contributing in adaptation of *P. tricornutum* to decadienal stress signals.

Introduction

Phytoplankton plays a prominent role as photosynthetic producers in the marine food chain. Diatoms are a dominant group of heterokont phytoplankton including around 200,000 species (Armburst et al., 2009) which are responsible not only for about 32% of global primary production (Falkowski et al., 1998; Uitz et al., 2010) to provide carbon for food webs but are also major players in atmospheric carbon cycling. Diatoms and other heterokonts are unique in their evolutionary history due to their origin as a result of a secondary endosymbiotic event wherein green alga and later red alga were acquired by a heterotrophic eukaryote (Bowler et al., 2010; Moustafa et al., 2009). A number of horizontal gene transfer events have led to unique genetic complement and distinct gene expression control mechanisms in these organisms (Allen et al., 2006) which help them adapt rapidly to the dynamic short time scale changes in ocean environment. Apart from the diatoms being unique in their origin and characteristic silica cell wall (Tirichine and Bowler, 2011), presence of metabolic pathways such glycolytic pathway, urea cycle, pathway for synthesis of sterols and mechanisms for carbon concentration have contributed to their great ecological success in oceans (Allen et al., 2011; Fabris et al., 2012, 2014; Prihoda et al., 2012).

Along with some other diatoms, the whole-genome sequencing of the two model diatoms *Cyclotella nana* (reported as *Thalassiosira pseudonana)* (Armbrust et al., 2004) and *Phaeodactylum tricornutum* (Bowler et al., 2008) have revealed important information on genome structure and organization and on various regulatory and metabolic pathways in these diatoms. Both the species have small genome sizes with *Cyclotella* (*Thalassiosira)* being 32.4 megabases (Mb) and *P. tricornutum* (CCMP2561), 27.4 Mb containing about 10000-14000 genes (Armbrust et al., 2004; Bowler et al., 2008; Mock et al., 2008; Maheswari et al., 2009). The analysis of genomes show that apart from the diatom specific genes, pool of genes from other organisms were acquired by horizontal gene transfer mechanisms leading to relatively little overlap between different diatom species. This might be a valid reason for species specific responses to changes in various environmental conditions like abiotic (nutrient, light, pH etc.) and biotic (grazing) stress.

Phaeodactylum tricornutum has become a model diatom due to its genome availability (with 10042 predicted genes) (Bowler et al., 2008), development of molecular tools for genetic studies (Siaut et al., 2007) and ease of growth. The availability of diatom genomes and advances in high-throughput sequencing techniques gives ample prospects to do genome wide transcriptomics studies which would add to the knowledge on gene level regulation of molecular and biochemical changes underlying flexibility and ecological success of these organisms under rapidly changing environmental conditions.

There has been number of recent transcriptomic studies that have suggested that various molecular and biochemical changes in response to environmental variations are regulated at gene level in diatoms. Chauton et al. (2013) showed that transcripts associated with carbohydrate and lipid metabolism are differentially regulated in *P. tricornutum* when grown in light-dark cycle. They suggested that carbon synthesis occurs in chloroplast during daytime whereas carbon rich compounds are broken down at dark in cytosol and mitochondria which are dependent on diurnal transcriptional regulation of genes involved

in tricarboxylic acid (TCA) cycle and fatty acid biosynthesis. Study done by Yang et al. (2013) to assess changes in *P. tricornutum* under nitrogen deprivation, which resulted in accumulation of neutral lipids were co-related to upregulation of genes involved in carbon fixation, TCA cycle and glycerolipid metabolism including nitrogen assimilation and downregulation of genes in photosynthesis. Yang et al. (2014) in yet another study to understand molecular and cellular responses of *P. tricornutum* to phosphorous limitation showed that genes were regulated at transcript level for adaptation of cells to phosphorous stress. Increased levels of genes associated with phosphorous uptake, stress shock response, protein translation, carbon fixation, glycolysis and TCA cycle whereas decline in genes associated with beta-oxidation were seen. These changes were in accordance with increased triacylglycerols, changes in fatty acid composition, ultrastructure membrane changes and other metabolic changes. Another recent study on nitrogen stress in *P. tricornutum* reported that lipid accumulation is a result of remodeling of intermediate metabolism mainly by transcriptional regulation of genes in urea cycle, TCA cycle, chlorophyll and lipid biosynthesis to help cells rapidly response to changes in nitrogen availability (Levitan et al., 2015). Alipanah et al. (2015) also integrated physiological data with transcriptome data during nitrogen deprivation in *P. tricornutum* cells. They reported that N-deprivation led to decreased in photosynthesis and chlorophyll content whereas increase in neutral lipids that were regulated by changes in transcript levels of genes involved in transport, assimilation, synthesis and cycling of nitrogen along with genes associated with photosynthesis and chlorophyll synthesis, glycolysis, Calvin cycle, TCA cycle and pyruvate metabolism. Studies done by Gwak et al. (2014) on photooxidative stress in *Haematococcus pluvalis* also suggests that transcriptomes and lipidomes go hand in hand to protect organism from high radiance stress. Formation of pigments (astaxanthin) and TAGs at the cost of reduction in chloroplast and photosynthetic glycolipids were regulated to a comparable extent by upregulation of transcripts involved in pigment synthesis pathways and in *de novo* fatty acid synthesis and TAG assembly pathways.

In contrast to many transcriptomics studies as discussed above that were focused on long term (48 hr-72 hr) exposure of cells to nutrient stress, Matthijs et al. (2016) reported early (<20 hours) transcriptome regulation of nitrogen stress response in *P. tricornutum*. They reported that differential regulation of genes in urea, TCA, lipid, chlorophyll and other metabolic pathways begins early with the onset of nitrogen stress. In addition, studies done by Ritter et al. (2014) and Dittami et al. (2009) *in Ectocarpus siliculosus* also focused on short term acclimation (4-8 hr) of the organism to abiotic stress such as Copper, NaCl and H_2O_2 and revealed that transcriptome undergoes extensive reprogramming for acclimation to these stresses.

Some abiotic (nutrient) but mainly biotic stress such as cell wall damage or wounding in many diatoms induces production of bioactive oxylipins, a major class of these oxylipins being polyunsaturated aldehydes (PUAs) as a defense mechanism that inhibit reproduction in grazers (Wichard 2005, 2007; Pohnert 2002; Ribalet et al., 2014; Ianora and Miralto, 2010; Ka Samba et al., 2014). One such model PUA is Decadienal (DD) that alongside inhibiting reproduction in grazers also induces various molecular and biochemical changes in diatoms (Vardi et al., 2006, 2008, van Creveld et al., 2015). In the diatom *P. tricornutum*, DD induces calcium dependent NO production and genome wide transcriptional changes (Leflaive J et al., 2009 and Vardi et al., 2006, 2008). These PUAs (DD and many aldehydes and oxygenated lipids) are produced from membrane phospholipids via transformation of C16 and C20 fatty acids in number of studied diatoms (Wichard et al., 2005, 2007).

In our earlier study, we determined the effects of decadienal on fatty acid levels and lipid classes in healthy *P. tricornutum* cells within short time scale (3 hr-6 hr). Our data showed that decadienal treatment resulted in decline in most fatty acid levels at 3 hr and 6 hr except increases in 18:1 and 18:2 at 6 hr. Analysis of lipid classes showed that there was no effect on abundant chloroplast glycolipids but increases in membrane phospholipids such as PE (1.8-2.7 fold) and PC (1.2 fold) and decline in PG (0.69 fold) and PS (0.36-0.28 fold).

There were small increases in non-polar (neutral lipids) (1.16-1.38 fold) whereas sterols showed an initial decline at 3 hr (0.86 fold) but remained unchanged at 6 hr (Chapter2). Further we wanted to understand the transcriptomic changes in response to sub-lethal DD dose within short time scale (3 hr-6 hr) in *P. tricornutum* with focus on lipid metabolism pathways.

We designed our experiments to mimic the situation of grazing (biotic) stress which induces release of bioactive oxylipins (PUAs and other oxidized lipids) from various diatoms in the marine environment. This was done with an aim to understand transcriptome regulation of lipid changes (Chapter 2) in the surrounding ungrazed or healthy diatoms in response to decadienal (DD). Global transcriptomic changes with focus on changes in lipid metabolism pathways were evaluated in the model diatom *P. tricornutum* in response to a 10µM sub-lethal dose of DD during early stationary phase of the growth.

Materials and Methods

Diatom strain, culture conditions and treatments

Phaeodactylum tricornutum CCMP2561 was obtained from Provasoli-Guillard National Center for Culture of Marine phytoplankton (CCMP), USA. The cultures were grown in F/2 medium with sterilized local seawater (30-34ppt) with inorganic nutrients and filter sterilized vitamins. Cultures were grown at 20^{o}C in a growth chamber equipped with cool-white fluorescent lights (80 µmol m^{-2} s^{-1}) on a 12:12 h light/dark cycle for 10-12 days and were periodically stirred by hand. Cells were treated with 10µM Decadienal (DD) (W313505; Sigma Aldrich) or DMSO (0.1% solvent control (276855, Sigma Aldrich) at stationary phase (2.5-3x10^6 cells/ml) and harvested by centrifugation for 10 minutes at 3500g for experiments. Decadienal treatments were done with cultures after 3 hr in their day cycle.

RNA isolation, library preparation and sequencing

For total RNA isolation, pelleted cells (after centrifugation) were resuspended in 500µl of PureLink ® Plant RNA reagent (Cat No. 12322-012, Life technologies) and transferred to 2ml Qiagen tissue lyser tubes (Cat No 990381, Qiagen). To each tube, 500 µl of 0.5mm Zirconia-silica beads (Cat. No. 11079105z, Biospec) were added and were put in Qiagen tissue lyser LT (bead beater) for mechanical tissue disruption and homogenization (50 oscillations/sec for two cycles of 2 min each, one cycle of 1 min with 30 secs rest intervals between each cycle). After homogenization, tubes were incubated horizontally for 5 mins and then centrifuged at 12000g for 2 mins. Supernatant was collected and transferred to 1.5 ml tubes to proceed with RNA isolation. RNA was isolated using protocol modified from manufacturer's protocol of small scale RNA isolation by PureLink ® Plant RNA Reagent (Life technologies). Additional washing steps with 75% ethanol were performed and finally RNA pellet was resuspended in 30µl RNAas free water. RNA concentration was measured using Nanodrop spectrophotometer and quality was analyzed by running RNA on denaturing gel.

Total RNA (5-9 µg) isolated from two biological replicates each of DMSO (0.1%) solvent control and 10µM DD treated cells at 3 hr and 6 hr time points (total 8 samples) were submitted to Genome Sequencing facility (GSAF) at University of Texas at Austin, USA for quality assessment using Bio Analyzer, library preparation and RNA sequencing using Illumina NextSeq 500 single end (1x75) platform. Library preparation for preparing samples for sequencing were done using Poly(A)Purist Magnetic Kit (Life Technologies) and NEBNext Module Components using Bead-In High-Throughput dUTP RNA-Seq Library prep protocol according to manufacturer's instructions.

RNA-Seq data analysis

After obtaining the raw reads from Illumina sequencing, low quality reads, overrepresented sequences and sequence duplication was checked and files were filtered for quality using fastqc. Since adaptor contamination was very minimal, adaptor trimming was not necessary. For mapping reads to the reference, GFF filtered models and genome files available at Ensembl (http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index/) and Joint Genome Institute website (http://genome.jgi.doe.gov/Phatr2/Phatr2.home.html) were used. Mapping and assembly were done using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml). Gene-level differential expression was evaluated using the negative binomial distribution-based method implemented by the DESeq2 bioconductor package (Love 2014). MA plots were generated using the plotMA function from the DESeq2 package. Principal components analysis (PCA) was applied to the log-transformed DESeq-normalized data. Data was centered in both row- and column-dimensions but otherwise unscaled prior to calculation of principal components via singular value decomposition (SVD) for biplot visualization (Wouters 2003).

Gene ontology (GO) analysis was conducted using the Mann-Whitney U test methodology described in (Voolstra 2011, Wright 2015) applied to the DESeq differential expression test results. This method tests the differential enrichment of a GO category by comparing the relative magnitudes of the set of individual-gene test statistics (or, equivalently, p-values) for genes annotated as in the category to those of genes not included in the GO category. While the resulting GO enrichment scores do not depend on any gene-level significance threshold, we do here summarize the results for the most enriched GO categories in figure 3.2 by specifying how many genes had DESeq test statistics greater than 2.75 (corresponding to false-discovery rate adjusted p-values less than approximately 0.01 in the 3 hour data set and 0.02 in the 6 hour data set). Cluster heatmaps were generated using the R package pheatmap using the McQuitty linkage method for hierarchical clustering.

Results and Discussion

High throughput analysis of differential gene expression

RNA-Sequencing data generated from biological replicates of DMSO (0.1%) solvent control (S1 and S2) at 3 hr, 10µM DD treated samples at 3 hr (S3 and S4), DMSO (0.1%) solvent control at 6 hr (S6 and S8) and 10µM DD treated samples at 6 hr (S5 and S7) were assembled and mapped yielding around 12321 genes at 3 hr and 6 hr corresponding to 97% of the *P tricornutum* coding genome (genome.jgi-psf.org/Phatr2). MA plots and Principal coordinate analysis (PCA) plots were run on raw data for overall visualization of RNA Seq gene expression data. MA plots for 3 hr and 6 hr samples (Figure 3.1a and 3.1b) showed that there were more gene expression changes (red dots indicates genes with varying expression) at 3 hr time point than 6 hr time point in response to DD treatment. Principal coordinate analysis of the variance-stabilized data for all genes (Figure 3.1c and 3.1d) where PC1 axis separated DMSO control versus DD treatment samples and PC2 axis separated time point 3 hr versus 6 hr samples revealed expression differences between the samples based on both treatment and time and also validated that there wasn't a significant variation among biological replicates of control or treated samples. DESeq analysis of data set showed that in response to DD, globally 7320 protein coding genes at 3 hr and 4631 genes at 6 hr were differentially expressed (p_{adj} or $q < 0.05$) when compared DMSO solvent control (data not shown). Among them, there were around 500 genes and 340 genes which were upregulated ($\log_2$FoldChange >1.2) at 3 hr and 6 hr respectively and about 4000-6000 genes downregulated (data not shown) indicating that the treatment of decadienal induced wide reprogramming of transcriptome in *P tricornutum*.

Gene Ontology (GO) and Heatmap analysis: processess affected in repsonse to DD

Gene Ontology (GO) enrichment analysis was done on both 3 hr and 6 hr time point DESeq dataset using Mann-Whitney U test and adaptive clustering method similar to as described

in Wright et al. (2015) and Voolstra et al. (2011). This analysis allowed general examination of biological and molecular processes most affected or differentially regulated in response to DD at 3 hr and 6 hr (Figure 3.2a and 3.2b). Most GO enriched terms or processes affected in response to DD treatment at 3hr were related to photosynthesis and light harvesting, generation of precursor metabolites and energy, protein catabolism, translation, ribosome biogenesis, carbohydrate metabolism and unsaturated fatty acid metabolic process. Among the GO terms affected at 6hr time point, significant ones were chromosome, chromatin and organelle organization and unsaturated fatty acid metabolic process. Interestingly the GO group 'unsaturated fatty acid metabolic process' was found to be significantly affected in response to DD treatment both at 3 hr and 6 hr time points. Figure 3.4 represents the heat-map of genes associated with top GO group 'generation of precursor metabolite and energy' at 3 hr time point, top GO group at 6 hr 'chromosome organization' and overlapping GO group both at 3 hr and 6 hr 'unsaturated fatty acid metabolic process' in DMSO control (C) and DD treated (T) at 3 hr and 6 hr. More than 90% of the genes in these GO processes were differentially regulated in DD treated samples when compared to DMSO solvent control both at 3 hr and 6 hr.

Analysis of upregulated and downregulated GO groups

Further detailed analysis of GO groups enriched in upregulated and downregulated genes were done for 3 hr and 6 hr in response to DD treatment. This was done to get more understanding on regulation of various processes in *P. tricornutum* cells in response to DD treatment. The processes in red indicated GO processes which were enriched in downregulated genes and that in blue indicated processes which were enriched in upregulated genes (Figure 3.3a and 3.3b). At 3 hr with DD treatment, processes related to photosynthesis, generation of metabolite and energy, various ion transports, inorganic anion transport, cation transport, phosphate ion transport, tetrapyrrole metabolic process, metabolic processes for porphyrin containing compounds and cofactors and chromosome organization were downregulated. Processes for RNA processing, RNA metabolic process,

macromolecule and protein catabolic processes, translation, protein folding, cellular lipid metabolic processes and cellular homeostasis were upregulated. At 6 hr with DD treatment, to an extent similar trend was seen wherein cation/anion, metal ion transport, nitrogen compound transport, various DNA metabolic process, chromatin/chromosome organization, regulation of cellular process, response to stimulus and macromolecule metabolic processes were downregulated. Quite similar to 3 hr, various RNA metabolic processes, RNA processing, various catabolic carbohydrate and protein processes, cellular lipid metabolic processes, carbohydrate and organic acid metabolic processes, amino acid biosynthesis, translation and translational initiation and cellular homeostasis were upregulated. This may suggest that in response to decadienal, cells may divert its resources and energy towards increased lipid and protein synthesis processes, post transcriptional and translational mechanisms downsizing photosynthesis, ion transport processes and DNA metabolism, chromatin/chromosome organization and cell cycle processes. This suggests that *P. tricornutum* responds to decadienal stress by extensive reprogramming of its transcriptome to adapt to rapidly changed environment in short time scale. Downregulation of photosynthesis and energy metabolism processes has been previously observed in nitrogen and phosphorous stress studies in *P. tricornutum* (Yang et al., 2014; Alipanah et al., 2015).

Analysis of genes in GO groups: 'Unsaturated fatty acid metabolic process'; 'phospholipid biosynthetic process' and 'lipid metabolism processes'

Among various GO groups affected in response to DD, an interest was to focus on lipid metabolism processes to understand transcriptome regulation of lipid changes observed in response to decadienal (Chapter 2). Out of various GO groups, three go groups related to lipid synthesis and metabolism i.e. unsaturated fatty acid metabolic process, phospholipid biosynthetic process and lipid metabolism process were sorted and genes under these processes were annotated using JGI genome source of *P. tricornutum* (http://genome.jgi.doe.gov/Phatr2/Phatr2.home.html). Table 3.1 and 3.2 gives list of

significant genes (padj or q <0.05) in these selected GO processes with their log fold changes in DD treated cells compared to DMSO solvent control at 3 hr and 6 hr respectively. There were a total of 56 genes at 3 hr and a total of 39 genes at 6 hr out of total 75 annotated genes which were differentially regulated in response to DD treatment in these GO categories. A heatmap of genes in these three GO categories (Figure 3.5) represents a large number of genes in cellular lipid metabolism processes being regulated at their transcriptome levels which may correlate to observed changes in lipid classes in response to decadienal (Chapter 2).

Most genes under GO category 'unsaturated fatty acid metabolic process' were upregulated in response to DD (Table 3.1 and 3.2) both at 3 hr and 6 hr. These are the genes involved in fatty acid biosynthesis process in *P. tricornutum*. PTD9 (delta 9 desaturase) and FAD2 (delta-12 desaturase) are responsible for production of 18:1 and 18:2 fatty acids (Domergue, 2003) which were upregulated by 4.09 and 2.9 fold at 3 hr and 2.4 and 2 fold at 6 hr. This correlates to our data on observed increases in measured 18:1 and 18:2 fatty acids (1.12 and 1.46 fold) in response to DD (Chapter 2) indicating that increased levels of these fatty acids may be due to increased gene expression of genes coding for PTD9 and FAD2 enzymes. Further PTD5a responsible for production of 20:5 fatty acid was upregulated by 2.3 and 1.8 fold at 3 hr and 6 hr but our lipid data showed decreases in 20:5 fatty acid levels (0.7-0.8 fold) (Chapter 2). This suggests involvement of possible translational and post translational modifications or rapid turnover or processing of 20:5 fatty acid into downstream products in response to decadienal as 20:5 fatty acid is a known substrate for synthesis of downstream oxylipins (Wichard, 2007). PTD6 was also upregulated by 1.46 and 1.54 fold at 3 hr and 6 hr, which is responsible for conversion of 18:2 to 18:3 (n-6 pathway) or 18:3 to 18:4 (n-3 pathway) (Domergue, 2002). Although most of the unsaturated fatty acid synthesis gene transcripts were upregulated, we observed a decline in most unsaturated fatty acids except 18:1 and 18:2 (which increased) that might indicate post translational regulation of these enzymes or peroxidation or utilization of

these fatty acids to other subsequent products. Almost all the genes in GO category 'phospholipid biosynthesis processes' were downregulated (Table 3.1 and 3.2) however we observed increases in PC (1.21 fold), PE (1.8- 2.7 fold) phospholipid classes and decline in PS (0.36-0.28 fold), PG (0.69 fold) and LPG (0.56 fold) classes in response to DD (Chapter 2). GPAT (glycerol-3-phosphate acyltransferase) was downregulated (0.27 fold) at 3 hr, which forms lysophosphatidic acid (Figures 1.8, 1.9). Transcript levels for enzyme LPAAT (lysophosphatidate acyltransferase) were also downregulated both at 3 hr and 6 hr (data not shown) that are responsible for production of PA (phosphatidic acid) (Figure 1.9). PAP (phosphatidic acid phosphatase) forms DAG (diacylglycerol) from PA. PAP was upregulated at 3 hr (1.3 fold) and downregulated (0.3 fold) at 6 hr. PA and DAG as well as other phospholipids can act as precursors for the production of sulfolipids and galactosylglycerides. Also transcript level of gene coding phosphoinositide phospholipase C involved in one of the steps for DAG formation from phosphoinositide (PI) was downregulated both at 3 hr and 6 hr. Another transcript for gene coding for phosphatidylcholine-sterol O-acyltransferase was downregulated at 3 hr, which is involved in glycerophospholipid metabolism indicating that might increase phospholipids compared to DAGs and TAGs. Overall this indicates that transcriptome in combination with post transcriptional and translational regulation might be responsible for observed lipid changes in *P. tricornutum* cells in response to DD. In contrast, Alipanah et al. (2015) in their study of N deprivation in *P. tricornutum* cells that induces TAG accumulation reported increases in upregulation of phospholipase C and D which indicated degradation of membrane phospholipids to provide precursors PA and DAG for TAG production. This supports our observed lipid changes in response to decadienal wherein there were slight increases in TAGs and changes in membrane lipids were more appealing. Also our data on transmission electron microscopy (TEM) images of DD treated cells didn't observe any degradation or disorganization of chloroplast membranes (data not shown) in contrast to the study of Yang et al. (2013) where nitrogen deprived cells showed disorganized or dispersed chloroplast

membranes and large oil bodies (TAGs) supporting accumulation of TAGs levels on expense of phospholipids.

Interestingly genes coding for enzymes such as ACC (acetyl CoA carboxylase), FAS (fatty acid synthase) and KAS (keto-acyl synthase) that are involved in initiating de novo fatty acid biosynthesis processes in chloroplast were downregulated at 3 hr. At 6 hr, two genes coding for KASII was upregulated by 2.1 and 1.5 fold. Between two acyl carrier proteins ACP, transcript levels of one were downregulated (0.28 fold at 3 hr, 0.32 fold at 6 hr) whereas not much of a change was seen in the second (1.01 fold at 3 hr and 1.07 fold at 6 hr). ACP is a universally highly conserved carrier of acyl intermediates during fatty acid synthesis. Downregulation of these genes might be reason for observed decline in free fatty acids at 3 hr in response to decadienal (Chapter 2). Alipanah et al. (2015) also reported downregulation of transcripts related to the chloroplast fatty acid biosynthetic pathway in response to N deprivation in *P. tricornutum* cells and corresponding lower levels of free fatty acids in the cells suggesting that this was due to their incorporation into TAGs.

Lipases are enzymes that de-esterify lipids such as TAGs and phospholipids and Yang et al. (2013) reported presence of 28 putative lipases in *P. tricornutum* genome. A number of TAG lipases (five at 3 hr; four at 6 hr) were mostly seen to be downregulated both at 3 hr and 6 hr except one lipase which was upregulated to 1.3 fold at 6 hr in response to DD. Alipanah et al. (2015) showed that in response to N deprivation in *P. tricornutum*, upregulation of lipases could be related to recycling of previous TAGs and synthesis of new TAGs or for modifications in TAG structure, thus playing a role in TAG accumulation. Our data does report significant but small increases (1.16-1.38 fold) in TAGs in response to DD (Chapter 2) along with increases in certain membrane phospholipids on the contrary to other studies of abiotic N and P limitation (Alipanah et al., 2015; Yang et. al, 2014) wherein TAG accumulation is major phenomenon occurring at cost of decline in membrane phospholipids. Hence observed decline in TAG lipases should not be very surprising. Yang

et al. (2013) reported that in their study of neutral lipid accumulation under N deprivation, eight lipases were upregulated whereas others were all downregulated suggesting that overall reduced mRNA levels of lipases would protect lipids (TAGs) from degradation promoting remodeling of TAG accumulation under N stress. Hence our observed downregulation of transcripts of lipases should help protect TAGs or other phospholipids which could be later used to remodel lipids to overcome decadienal stress.

A number of genes involved in isoprenoid biosynthesis pathways and carotenoid biosynthesis pathways were also downregulated (Table 3.1 and 3.2) indicating that carbon resources were restructured or importantly allocated to processes required to survive or adapt to decadienal stress instead of synthesis of isoprenoids or accumulation of pigments.

Also, transcript levels of enzyme involved in lipid catabolism, acetyl co-A oxidase was downregulated to 0.47 and 0.58 fold at 3 hr and 6 hr in response to DD. Yang et al. (2013) also showed reduced levels of transcripts of enzymes acyl-CoA oxidase and 3-oxoacyl-CoA thiolase under N deprivation which was in accordance with increased transcript levels of enzymes in TAG synthesis hence promoting lipid accumulation. They showed increase in transcript levels of enzyme PDAT (phospholipid: diacylglycerol acyltransferase) involved in last step of TAG synthesis whereas downregulation of this enzyme was seen at 3 hr with DD treatment in our study. This might indicate that not very high increases in TAGs in our study could be due to downregulation of PDAT enzyme along with downregulated lipases and oxidases that help accumulate or protect TAGs in the cells.

Two transcripts for a gene encoding cyclopropane-fatty-acyl-phospholipid synthase were seen to be differentially regulated. One transcript was upregulated to 1.43 fold both at 3 hr and 6 hr, however another transcript was downregulated 0.65 and 0.46 fold at 3 hr and 6 hr in response to decadienal. This enzyme is known to be involved in synthesis of cyclopropane fatty acids (CFA) from unsaturated fatty acid (UFA) phospholipids. Some

interesting studies have been done in E.coli where in CFA phospholipids accumulate in the cells during early stationary phase (Grogan et al., 1997). The physiological role of CFA phospholipids have been predicted to be in temporal energy storage and membrane fluidity modification. However, it was also reported that CFAs are more stable and less reactive that UFA to oxidations by ozone or singlet oxygen, hence providing membrane stability (Law, 1971). Harley et al., (1978) proposed that CFAs maintain membrane properties which are beneficial for cell survival. Hence DD induced regulation of CFA synthases may be either to provide an alternate energy storage or more importantly in modifying membrane properties to overcome DD stress. Since our earlier data on membrane permeability assays showed altered permeability to certain stains (Chapter 2), this could indicate a possible role of CFA in modifying membrane structure to alter its permeability to selective molecules.

Top upregulated and downregulated genes in response to DD

Among the top genes which were upregulated to 4-7 folds in response to DD at 3 hr were NADH oxidase, genes involved in cell cycle, cell division and chromosome partitioning, heat shock protein *Hsp20,* ABC transporter like and methionine sulfoxide reductase (some genes have no annotation available yet)(Figure 3.6). At 6 hr, similar genes were seen to be upregulated which included genes in cell cycle/cell division, NADH oxidase, Zn containing alcohol dehydrogenase, Glutathione S transferase and ABC transporter like genes (some genes have no annotation available yet) (Figure 3.6). Most of these genes are involved in oxidative stress regulation or antioxidant defense mechanisms, generation of superoxide ions, breakdown of aldehydes or metabolites into useful or less toxic products and detoxification mechanisms. Since it is known that DD induces NO and other ROS production in *P. tricornutum* (Vardi et al., 2006), it is not surprising that these genes are among the top upregulated genes to regulate ROS stress in response to decadienal. Studies done in *Thalassiosira pseudonana* (Thamatrakoln et al., 2012) also showed a regulation of

similar group of genes in response to iron induced oxidative stress in co-ordination with other metabolic pathways for ecological adaptation to changed environment. Rossenwasser et al. (2014) identified approximately 300 redox sensitive proteins mediating response of nitrogen stress induced oxidative stress in *P. tricornutum* indicating that transcriptome and proteome work in cohesion to determine cell fate in response to environmental stress. Van Creveld et al. (2015) reported that mitochondrial glutathione (GSH) redox potential determines cell fate in the diatom *P. tricornutum* in response to DD. Hence observed upregulation of glutathione transferase gene among top upregulated genes in our study may be to help regulate glutathione levels to maintain ROS levels and acclimate to DD induced oxidative stress.

Among the top downregulated genes (-7 to -4 fold) at 3 hr were genes such as nitrate reductase, ferric reductase, possible nitrate transporter, glycosyl transferase and genes for proteins in chromosome condensation complex (some genes have no annotation available yet) (Figure 3.7). At 6 hr, were genes such Myb DNA binding, nuclear protein SET, Barren (mitotic cell cycle) and carbonic anhydrase (some genes have not been annotated yet) (Figure 3.7). This shows that cells regulate C and N metabolism in response to decadienal stress. This correlates well to downregulation of ion transport, DNA metabolic processes GO groups as discussed earlier (Figure 3.3). Alipanah et al. (2015) in their study of N deprivation stress in *P. tricornutum* reported upregulation of nitrate transporter, nitrate reductase and downregulation of carbonic anhydrase genes to increase uptake/ availability of nitrogen , amino acids and carbon sources to maintain N and C metabolism pathways. Downregulation of genes involved in ion transport mechanisms observed in our study is logical as cells do not need to spend energy in transport mechanisms and instead regulate processes to help with DD stress.

Conclusions

Genome wide transcriptome studies in various diatoms including those in *P. tricornutum* have provided useful mechanistic information on regulation of various processes in these diatoms supporting their ecological adaptation to continuous changing environments. These organisms are useful sources of lipids including PUFAs hence it is important to understand regulation of lipid metabolic processes under the conditions of stress. Although various studies on abiotic stress (nutrient limitation being a major one) have been done, still limited knowledge is available on biotic stress such as grazing or that of the effect of oxylipins including PUAs that are released from diatoms upon cell damage. Our earlier studies reported changes in fatty acids and polar and non-polar lipid classes in response to sublethal dose of decadienal in healthy *P. tricornutum* cells within short time scale. This report attempts to understand underlying overall general transcriptome regulation of various processes under same sub lethal DD dose within short time scale with more focus on lipid metabolic processes. Overall it was seen that photosynthesis, ion transport mechanisms, DNA metabolic processes and chromosome/chromatin organization processes were downregulated whereas processes relating to RNA metabolism, carbohydrate and protein metabolism, translation, amino acid synthesis and cellular lipid metabolic processes were upregulated. Within lipid metabolic processes most of the genes involved in initiating fatty acid synthesis in chloroplast were downregulated but many genes involved in synthesis of PUFAs via combination of omega-3 and omega-6 pathways in *P. tricornutum* were upregulated. Also few genes involved in TAG synthesis were downregulated. Genes encoding enzymes that catalyze lipid degradation and oxidation were also downregulated, which would help minimize loss of lipids in the cells. An analysis of top upregulated genes revealed that these included those involved in antioxidant defense mechanisms, detoxification mechanisms and cell cycle control. Among top downregulated genes were those involved in nitrogen and carbon metabolism and genes coding proteins for DNA/chromosome binding indicating that cells in response to DD regulate various

processes to store energy and resources to divert them in maintaining biological functions for survival.

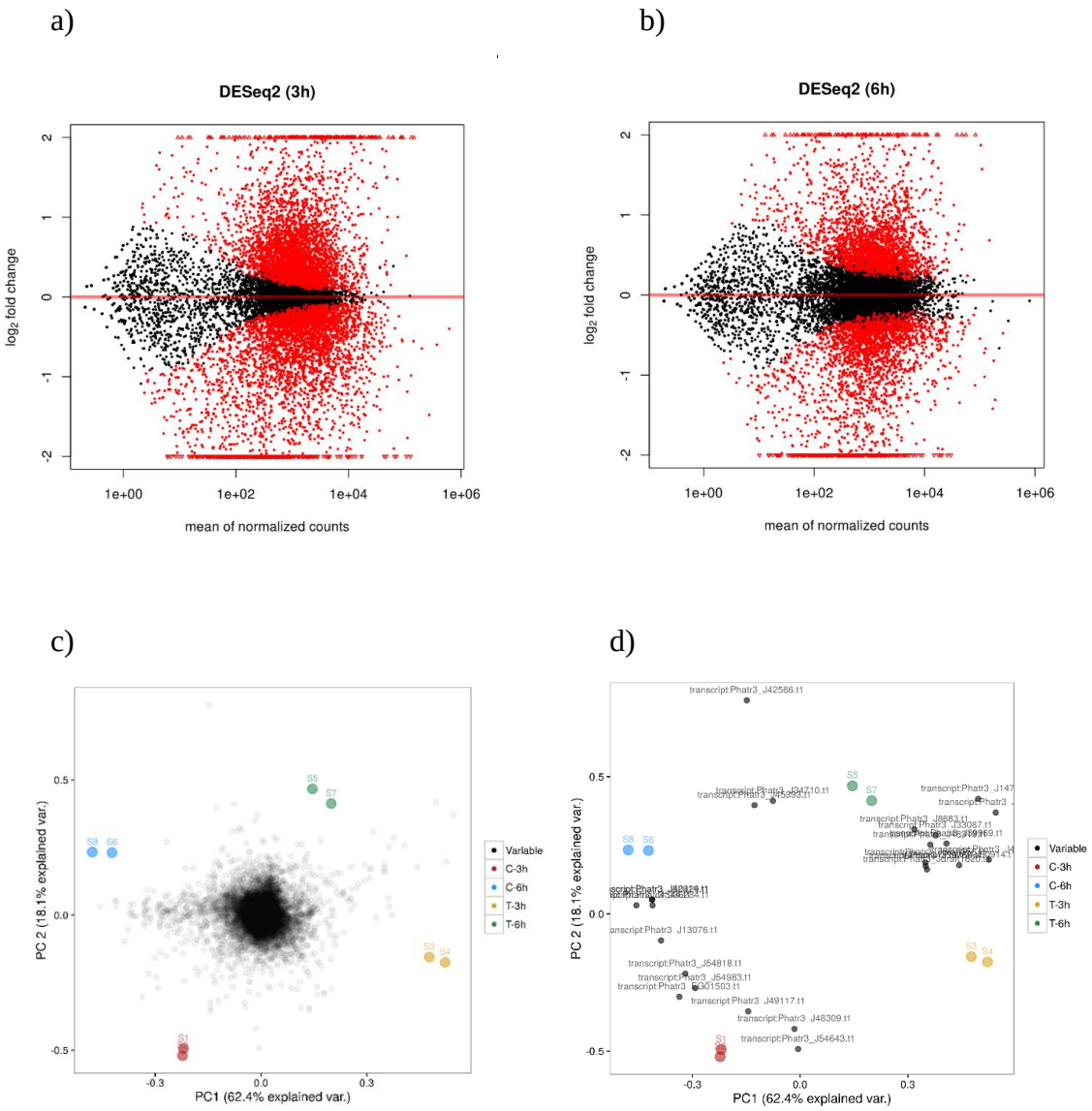
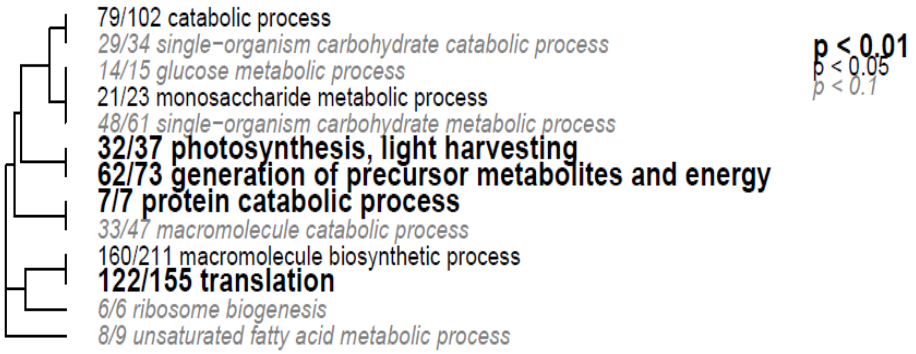


Figure 3.1: MA plots and Principal component analysis (PCA) plots based on DESeq results of 3 hr and 6 hr DD treated samples a)MA plot of DD treatment for 3 hr b) MA plot for DD treatment for 6 hr c) PCA plot showing all genes and d) PCA plot with labeled genes having high principal component.

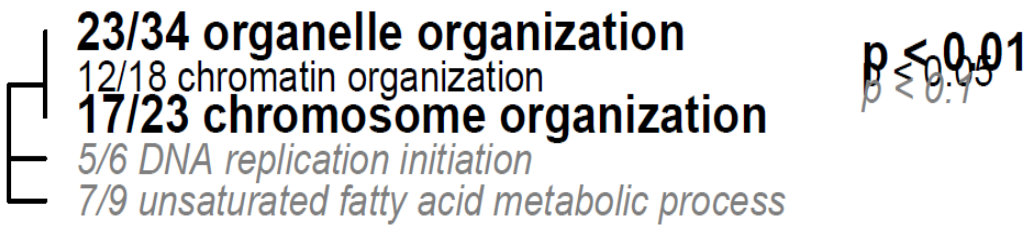
a)



pval	n	term(s)	name	p.adj
5 (-08)	73	GO:0006091	generation of precursor metabolites and energy	1.0 (-05)
1.4 (-07)	37	GO:0009765	photosynthesis, light harvesting	1.4 (-05)
0.00004	155	GO:0006412	translation	0.003
0.00015	7	GO:0030163	protein catabolic process	0.007
0.00027	211	GO:0034645 GO:0009059	macromolecule biosynthetic process	0.010
0.00033	102	GO:1901575 GO:0009056	catabolic process	0.011
0.00089	23	GO:0005996 GO:0019318	monosaccharide metabolic process	0.025
0.00235	15	GO:0006006	glucose metabolic process	0.057
0.00442	34	GO:0006096 GO:0044724 GO:0016052	single-organism carbohydrate catabolic process	0.092
0.00470	9	GO:0006636 GO:0033559	unsaturated fatty acid metabolic process	0.092
0.00525	61	GO:0044723	single-organism carbohydrate metabolic process	0.094
0.00618	47	GO:0006511 GO:0019941 GO:0043632 GO:0051603 GO:0044265 GO:0009057	macromolecule catabolic process	0.098
0.00647	6	GO:0042254 GO:0022613 GO:0044085	ribosome biogenesis	0.098

Figure 3.2 continued next page…

b)



pval	n	term(s)	name	p.adj
5.2 (-06)	23	GO:0051276	chromosome organization	0.001
3.3 (-05)	34	GO:0006996	organelle organization	0.003
0.0007	18	GO:0006334	chromatin organization	0.049
		GO:0034728		
		GO:0065004		
		GO:0006325		
		GO:0071824		
0.0019	6	GO:0006270	DNA replication initiation	0.093
0.0025	9	GO:0006636	unsaturated fatty acid metabolic process	0.098
		GO:0033559		

Figure 3.2: Gene Ontology analysis (GO) with mann-Whitney U test (MWU) and adaptive clustering for a) DD treatment for 3 hr and b) DD treatment for 6 hr. Gene ontology categories enriched by genes differential regulated in DD treated compared to DMSO solvent control samples. The size of the font indicates the significance of the term as indicated by the inset key. The fraction preceding the GO term indicates the number of genes annotated with the term that pass an adjusted p-value threshold of 0.05. The trees indicate sharing of genes among GO categories (the categories with no branch length between them are subsets of each other).

a)

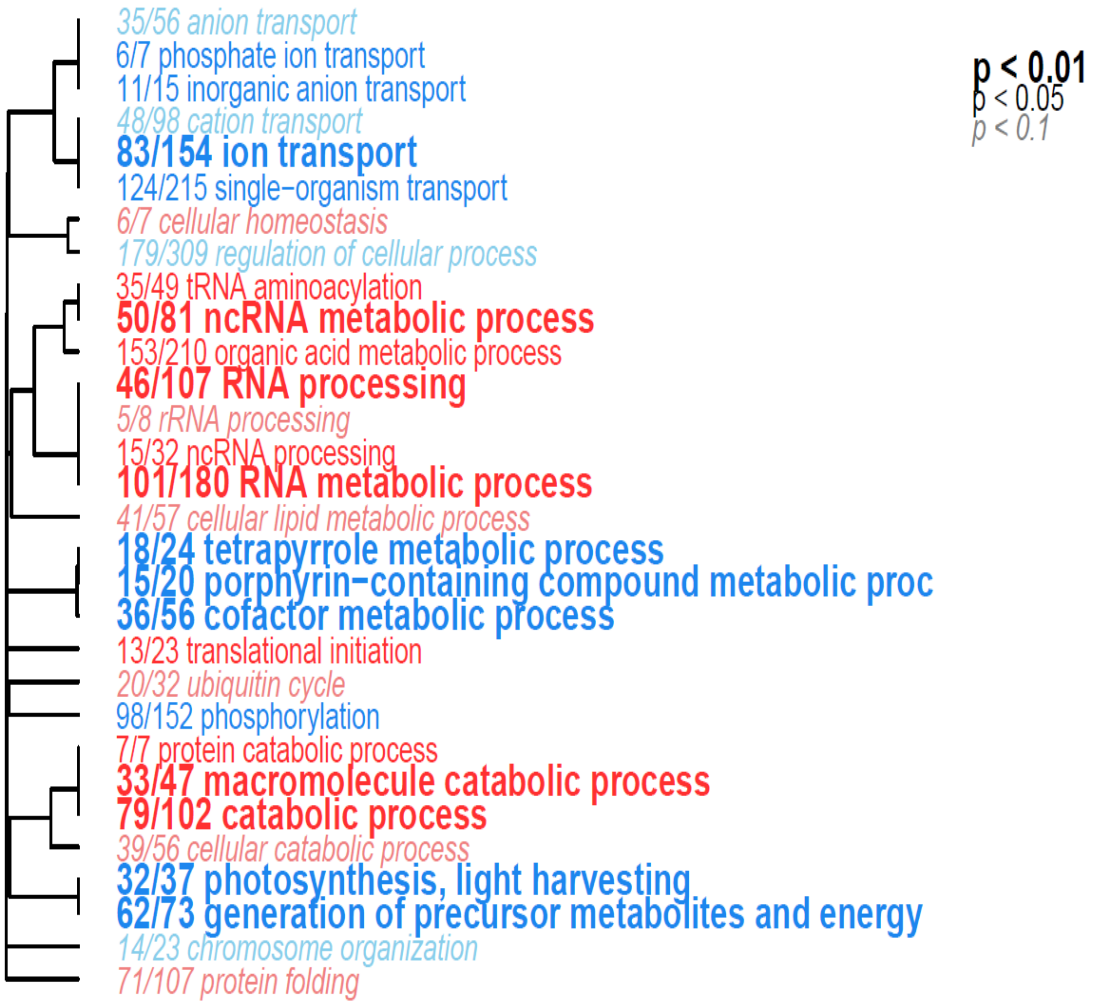


Figure 3.3: continued next page.

b)

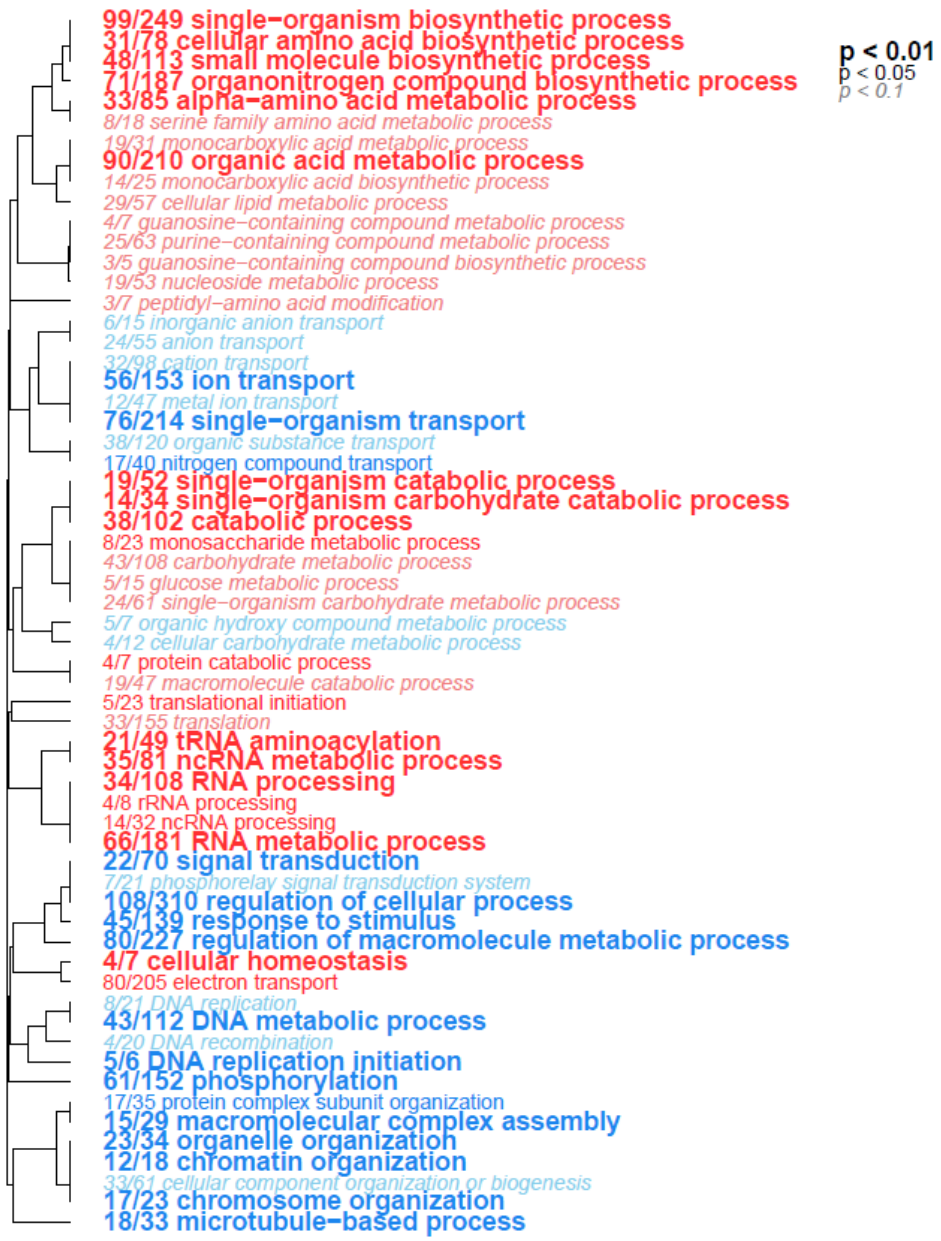


Figure 3.3: GO analysis of significantly regulated genes at a) 3 hr b) 6 hr of DD treatment. The dataset was divided into up- and downregulated genes and analyzed for process GO terms. The processes in blue indicates GO group enriched in downregulated genes and processes in red indicates GO groups enriched in upregulated genes.

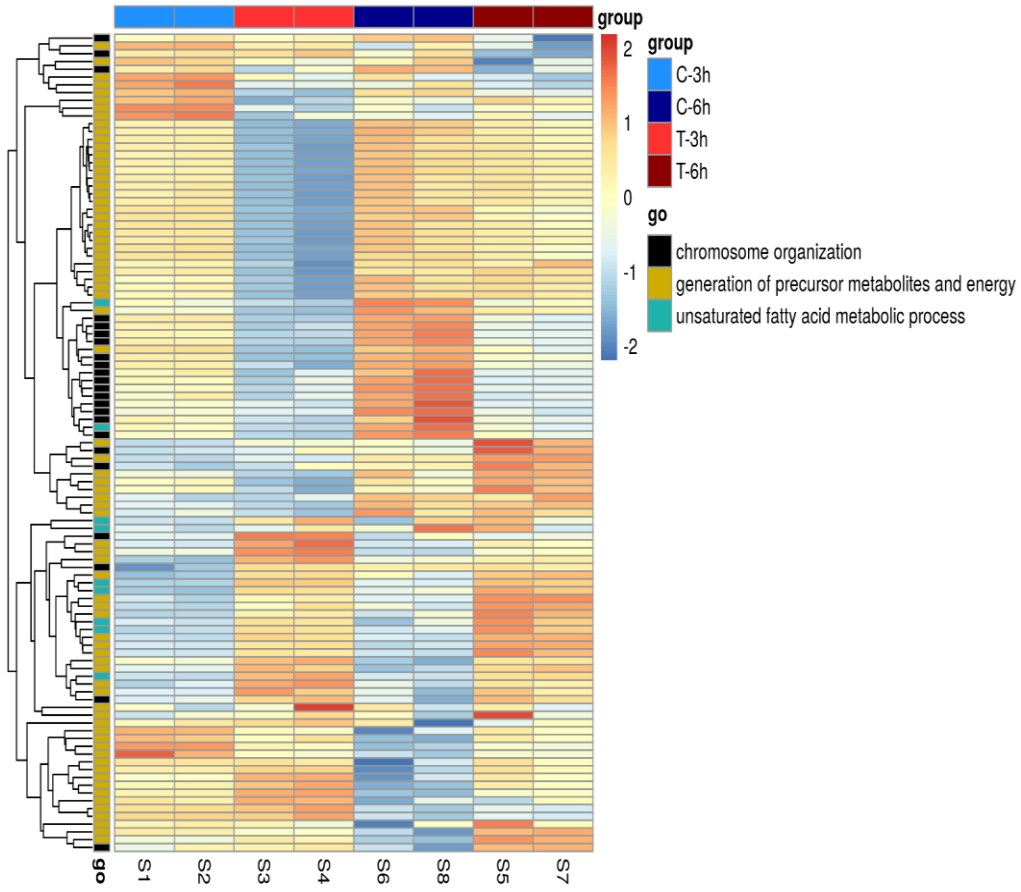


Figure 3.4: Heatmap of genes associated with top GO group and overlapping GO group in DMSO control (C) and DD treated (T) at 3 hr and 6 hr. (S1 and S2 -- DMSO control replicates at 3 hr; S3 and S4—DD treated replicates at 3 hr; S6 and S8—DMSO control replicates at 6 hr; S5 and S7—DD treated replicates at 6 hr)

Table 3.1: Differentially regulated genes (fold change), transcript ID and their annotation from JGI at 3 hr with DD treatment in GO categories 'unsaturated fatty acid metabolic process', 'phospholipid biosynthetic process' and 'lipid metabolic process'

GO group	JGI gene ID	log_2Fold Change	padj or q	Gene Name (Annotation from JGI)
unsaturated fatty acid metabolic process	transcript:Phatr3_J22510.t1	0.89	3E-49	Cytb5/FA desaturase?
unsaturated fatty acid metabolic process	transcript:Phatr3_J48423.t1	0.93	1.9E-56	Putative omega-6 desaturase, microsomal precursor/PTFAD6/PTD12
unsaturated fatty acid metabolic process	transcript:Phatr3_J29488.t1	1.46	1.5E-78	delta 6 fatty acid desaturase/PTD6
unsaturated fatty acid metabolic process	transcript:Phatr3_J22677.t1	-1.04	1.2E-26	putative dihydroderamide delta-4 desaturase/putative delta-4 desaturase involved in the desaturation of sphingolipids
unsaturated fatty acid metabolic process	transcript:Phatr3_J22459.t1	0.29	2.6E-05	delta 5 fatty acid desaturase /PTD5b
unsaturated fatty acid metabolic process	transcript:Phatr3_J46830.t1	2.36	0	delta 5 fatty acid desaturase/PTD5a
unsaturated fatty acid metabolic process	transcript:Phatr3_J25769.t1	2.99	8E-297	delta 12 fatty acid desaturase/FAD2
unsaturated fatty acid metabolic process	transcript:Phatr3_J46275.t1	-1.69	0.00015	Delta 6-fatty acid desaturase/delta-8 sphingolipid desaturase
phospholipid biosynthetic process	transcript:Phatr3_J33864.t1	1.39	1.2E-40	CDP-alcohol phosphatidyltransferase
phospholipid biosynthetic process	transcript:Phatr3_J45120.t1	0.70	1.2E-30	Myo-inositol-1-phosphate synthase
phospholipid biosynthetic process	transcript:Phatr3_J46136.t1	0.41	1.6E-05	Phosphatidate cytidylyltransferase
phospholipid biosynthetic process	transcript:Phatr3_J7678.t1	0.20	0.00061	phosphatidate cytidylyltransferase activity
phospholipid biosynthetic process	transcript:Phatr3_J13741.t1	0.58	2.2E-19	GPI transamidase subunit PIG-U
phospholipid biosynthetic process	transcript:Phatr3_J11916.t1	0.27	0.00088	Phospholipid/glycerol acyltransferase

Table 3.1 continued…

phospholipid biosynthetic process	transcript:Phatr3_J41845.t1	-0.32	1E-30	LytB / isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway/ HDR hydroxymethylbutenyl diphosphate reductase, chloroplast precursor
phospholipid biosynthetic process	transcript:Phatr3_J8663.t1	0.34	0.00044	CDP-alcohol phosphatidyltransferase
lipid metabolic process	transcript:Phatr3_J44231.t1	-0.29	1E-08	Lipase, class 3/Triglyceride lipases are lipases that hydrolyse ester linkages of triglycerides
lipid metabolic process	transcript:Phatr3_J19979.t1	0.47	5.5E-18	Acyl-CoA oxidase, peroxisomal precursor/ First step of peroxisomal fatty acid beta-oxidation
lipid metabolic process	transcript:Phatr3_J1174.t1	0.17	0.03691	Glycosyl transferase, family 28/lipid glycosylation; Covalent attachment of a glycosyl residue to a lipid molecule.
lipid metabolic process	transcript:Phatr3_J37367.t1	0.56	3.3E-12	(KAS II) /3-oxoacyl-[acyl-carrier-protein] synthase II (Beta-ketoacyl-ACP synthase II)
lipid metabolic process	transcript:Phatr3_J43463.t1	-0.39	1.2E-12	Lipase, class 3/ triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J49771.t1	-0.27	6.3E-12	phosphoinositide phospholipase C activity
lipid metabolic process	transcript:Phatr3_J48517.t1	0.65	4.1E-49	Cyclopropane-fatty-acyl-phospholipid synthase
lipid metabolic process	transcript:Phatr3_J39681.t1	0.24	6.8E-05	3-hydroxyacyl-Coenzyme A dehydrogenase, putative
lipid metabolic process	transcript:Phatr3_J31440.t1	0.29	2.9E-05	Acyl Carrier Protein (ACP), putative mitochondrial precursor.
lipid metabolic process	transcript:Phatr3_J44028.t1	0.47	5.2E-35	Lipase, class 3/triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J8860.t1	0.28	0.00035	phosphatidylcholine-sterol O-acyltransferase activity
lipid metabolic process	transcript:Phatr3_J15180.t1	0.97	1.8E-34	Polyprenyl synthetase/isoprenois biosynthetic process
lipid metabolic process	transcript:Phatr3_J12330.t1	0.26	0.0081	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, core/ terpenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J45886.t1	-1.17	3.9E-19	Carboxyl transferase/Acetyl-CoA carboxylase carboxyl transferase, beta subunit
lipid metabolic process	transcript:Phatr3_J50076.t1	-0.11	0.0497	Tyrosine specific protein phosphatase and dual specificity protein phosphatase
lipid metabolic process	transcript:Phatr3_J44955.t1	-0.90	3.7E-39	IspG protein/terpenoid biosynthesis process/1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; chloroplast precursor

Table 3.1 continued…

lipid metabolic process	transcript:Phatr3_J47271.t1	0.18	0.02706	Polyprenyl synthetase/isoprenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J9210.t1	-0.83	1.1E-37	CRTISO5 carotenoid isomerase 5, putative, phytoene dehydrogenase-related protein/carotenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J41624.t1	-0.41	3.6E-05	Esterase/lipase/thioesterase/Lipase class 3/ triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J50397.t1	0.33	7.4E-12	Esterase/lipase/thioesterase/Lipase class 3/ triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J19000.t1	-0.96	1.4E-15	Polyprenyl synthetase/isprenoid biosynthetic process/ farnesyltranstransferase. geranylgeranyl-diphosphate synthase
lipid metabolic process	transcript:Phatr3_J20120.t1	-0.42	9E-07	Beta-ketoacyl synthase/results in the formation of acetoacetyl ACP//Predicted UDP-galactose transporter
lipid metabolic process	transcript:Phatr3_J48864.t1	-0.89	9E-105	3-beta hydroxysteroid dehydrogenase/isomerase/steroid biosynthetic process
lipid metabolic process	transcript:Phatr3_J28797.t1	4.09	0	Fatty acid desaturase, type 1/ delta 9 desaturase, microsomal/ PTD9
lipid metabolic process	transcript:Phatr3_J45243.t1	0.60	0.00015	CRTISO4/ putative carotenoid isomerase; phytoene dehydrogenase-related protein
lipid metabolic process	transcript:Phatr3_J9258.t1	-0.66	4.5E-36	1-deoxy-D-xylulose 5-phosphate reductoisomerase/ isoprenoid/terpenoid synthesis process
lipid metabolic process	transcript:Phatr3_J29633.t1	1.43	5E-146	Cyclopropane-fatty-acyl-phospholipid synthase/ Cyclopropanation may contribute to the structural integrity of the cell wall complex
lipid metabolic process	transcript:Phatr3_J49702.t1	-0.35	0.01342	Lecithin:cholesterol acyltransferase/LACT/phospholipid:diacyl glycerol acyltransferase (PDAT)(EC:2.3.1.158), which is involved in triacylglycerol formation by an acyl-CoA independent pathway.
lipid metabolic process	transcript:Phatr3_J42917.t1	0.22	0.00283	Proteinase inhibitor I4, serpin/ Esterase/lipase/thioesterase
lipid metabolic process	transcript:Phatr3_J18940.t1	0.48	1.9E-06	Beta-ketoacyl synthase/ 3-oxoacyl-[acyl-carrier-protein] synthase II (Beta-ketoacyl-ACP synthase II) (KAS II)
lipid metabolic process	transcript:Phatr3_J49325.t1	-0.77	1.9E-23	Polyprenyl synthetase/isoprenoid biosynthetic process

Table 3.1 continued…

lipid metabolic process	transcript:Phatr3_J16615.t1	1.69	5.2E-41	Polyprenyl synthetase/isoprenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J12533.t1	-1.07	2E-177	NUDIX hydrolase, core/ Isopentenyl-diphosphate delta-isomerase/ isoprenoid synthesis pathway
lipid metabolic process	transcript:Phatr3_J21829.t1	-0.19	0.04838	4-diphosphocytidyl-2C-methyl-D-erythritol synthase/ isoprenoid biosynthesis process
lipid metabolic process	transcript:Phatr3_J35240.t1	-0.23	0.00355	Enoyl-CoA hydratase/isomerase/
lipid metabolic process	transcript:Phatr3_J9709.t1	1.02	2.3E-20	Acyl carrier protein (ACP)
lipid metabolic process	transcript:Phatr3_J16870.t1	-0.71	6.1E-63	Hydroxymethylglutaryl-CoA reductase, class I/II, catalytic/ synthesis of isoprenoids
lipid metabolic process	transcript:Phatr3_J9316.t1	1.68	0	Fatty acid desaturase, type 2
lipid metabolic process	transcript:Phatr3_J47049.t1	0.28	0.04253	Beta-ketoacyl synthase
lipid metabolic process	transcript:Phatr3_J43904.t1	0.85	4.5E-17	Phytoene dehydrogenase-related protein/ caretenoid biosynthesis

Table 3.2: Differentially regulated genes (fold change), transcript ID and their annotation from JGI at 6 hr with DD treatment in GO categories 'unsaturated fatty acid metabolic process', 'phospholipid biosynthetic process' and 'lipid metabolic process'

GO group	JGI gene ID	log_2Fold Change	Padj or q	Gene Name (annotated from JGI)
unsaturated fatty acid metabolic process	transcript:Phatr3_J22510.t1	0.67	1.55823E-10	Cytb5/FA desaturase?
unsaturated fatty acid metabolic process	transcript:Phatr3_J48423.t1	1.18	3.69662E-06	Putative omega-6 desaturase, microsomal precursor/PTFAD6/PTD12
unsaturated fatty acid metabolic process	transcript:Phatr3_J29488.t1	1.55	3.12806E-14	delta 6 fatty acid desaturase/PTD6
unsaturated fatty acid metabolic process	transcript:Phatr3_J22677.t1	-1.72	1.65808E-13	putative dihydroderamide delta-4 desaturase/putative delta-4 desaturase involved in the desaturation of sphingolipids
unsaturated fatty acid metabolic process	transcript:Phatr3_J46830.t1	1.80	9.92151E-42	delta 5 fatty acid desaturase/PTD5a
unsaturated fatty acid metabolic process	transcript:Phatr3_J25769.t1	2.02	7.47604E-19	delta 12 fatty acid desaturase/FAD2
unsaturated fatty acid metabolic process	transcript:Phatr3_J46275.t1	-3.38	1.37617E-43	Delta 6-fatty acid desaturase/delta-8 sphingolipid desaturase
phospholipid biosynthetic process	transcript:Phatr3_J33864.t1	0.42	0.015330446	CDP-alcohol phosphatidyltransferase
phospholipid biosynthetic process	transcript:Phatr3_J7678.t1	0.24	0.046165951	phosphatidate cytidylyltransferase activity
phospholipid biosynthetic process	transcript:Phatr3_J43010.t1	0.36	0.024863485	CDP-alcohol phosphatidyltransferase
phospholipid biosynthetic process	transcript:Phatr3_J47250.t1	-0.38	0.017212556	Phosphatidate cytidylyltransferase
phospholipid biosynthetic process	transcript:Phatr3_J37086.t1	-0.44	0.001458528	CDP-alcohol phosphatidyltransferase
lipid metabolic process	transcript:Phatr3_J44231.t1	-0.51	0.000117491	Lipase, class 3/Triglyceride lipases are lipases that hydrolyse ester linkages of triglycerides
lipid metabolic process	transcript:Phatr3_J19979.t1	0.58	2.19688E-06	Acyl-CoA oxidase, peroxisomal precursor/ First step of peroxisomal fatty acid beta-oxidation
lipid metabolic process	transcript:Phatr3_J10799.t1	0.34	0.002998251	Malonyl-CoA decarboxylase
lipid metabolic process	transcript:Phatr3_J37367.t1	2.17	2.44471E-21	(KAS II) /3-oxoacyl-[acyl-carrier-protein] synthase II (Beta-ketoacyl-ACP synthase II)

Table 3.2 continued…

lipid metabolic process	transcript:Phatr3_J49771.t1	-0.78	0.000004345	phosphoinositide phospholipase C activity
lipid metabolic process	transcript:Phatr3_J48517.t1	0.46	3.50764E-06	Cyclopropane-fatty-acyl-phospholipid synthase
lipid metabolic process	transcript:Phatr3_J39681.t1	0.34	0.004136623	3-hydroxyacyl-Coenzyme A dehydrogenase, putative
lipid metabolic process	transcript:Phatr3_J31440.t1	0.32	0.049583271	Acyl Carrier Protein (ACP), putative mitochondrial precursor.
lipid metabolic process	transcript:Phatr3_J44028.t1	1.36	2.91259E-10	Lipase, class 3/triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J15180.t1	0.58	1.70982E-06	Polyprenyl synthetase/isoprenois biosynthetic process
lipid metabolic process	transcript:Phatr3_J44955.t1	-0.34	0.006374245	IspG protein/terpenoid biosynthesis process/1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; chloroplast precursor
lipid metabolic process	transcript:Phatr3_J37711.t1	-0.30	0.031367645	Lipase, class 3 / triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J20120.t1	-0.77	9.54447E-05	Beta-ketoacyl synthase/results in the formation of acetoacetyl ACP//Predicted UDP-galactose transporter
lipid metabolic process	transcript:Phatr3_J18029.t1	0.33	0.016738952	Lipoate synthase/ lipoate biosynthetic process
lipid metabolic process	transcript:Phatr3_J28797.t1	2.45	7.72162E-30	Fatty acid desaturase, type 1/ delta 9 desaturase, microsomal/ PTD9
lipid metabolic process	transcript:Phatr3_J45243.t1	1.26	5.15067E-14	CRTISO4/ putative carotenoid isomerase; phytoene dehydrogenase-related protein
lipid metabolic process	transcript:Phatr3_J9258.t1	-0.47	0.000732427	1-deoxy-D-xylulose 5-phosphate reductoisomerase/ isoprenoid/terpenoid synthesis process
lipid metabolic process	transcript:Phatr3_J29633.t1	1.42	9.15569E-28	Cyclopropane-fatty-acyl-phospholipid synthase/ Cyclopropanation may contribute to the structural integrity of the cell wall complex
lipid metabolic process	transcript:Phatr3_J49325.t1	-1.19	1.75526E-07	Polyprenyl synthetase/isoprenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J16615.t1	0.98	2.77723E-06	Polyprenyl synthetase/isoprenoid biosynthetic process
lipid metabolic process	transcript:Phatr3_J35240.t1	-0.45	0.000193796	Enoyl-CoA hydratase/isomerase/
lipid metabolic process	transcript:Phatr3_J37652.t1	1.52	0.000190694	malonyl-CoA:ACP transacylase/ FASII/ Catalyzes the transfer of malonyl-CoA to malonyl-ACP. A subunit of the type II, plastid-targeted FAS. Probably contains a bipartite plastid targeting signal
lipid metabolic process	transcript:Phatr3_J47445.t1	-0.31	0.029402953	Lipase, class 3/ triglyceride lipase activity
lipid metabolic process	transcript:Phatr3_J9709.t1	1.08	4.22437E-07	Acyl carrier protein (ACP)

Table 3.2 continued…

lipid metabolic process	transcript:Phatr3_J16870.t1	-0.65	9.05986E-09	Hydroxymethylglutaryl-CoA reductase, class I/II, catalytic/ synthesis of isoprenoids
lipid metabolic process	transcript:Phatr3_J9316.t1	1.59	1.69583E-17	Fatty acid desaturase, type 2
lipid metabolic process	transcript:Phatr3_J43904.t1	0.74	8.19986E-10	Phytoene dehydrogenase-related protein/ caretenoid biosynthesis

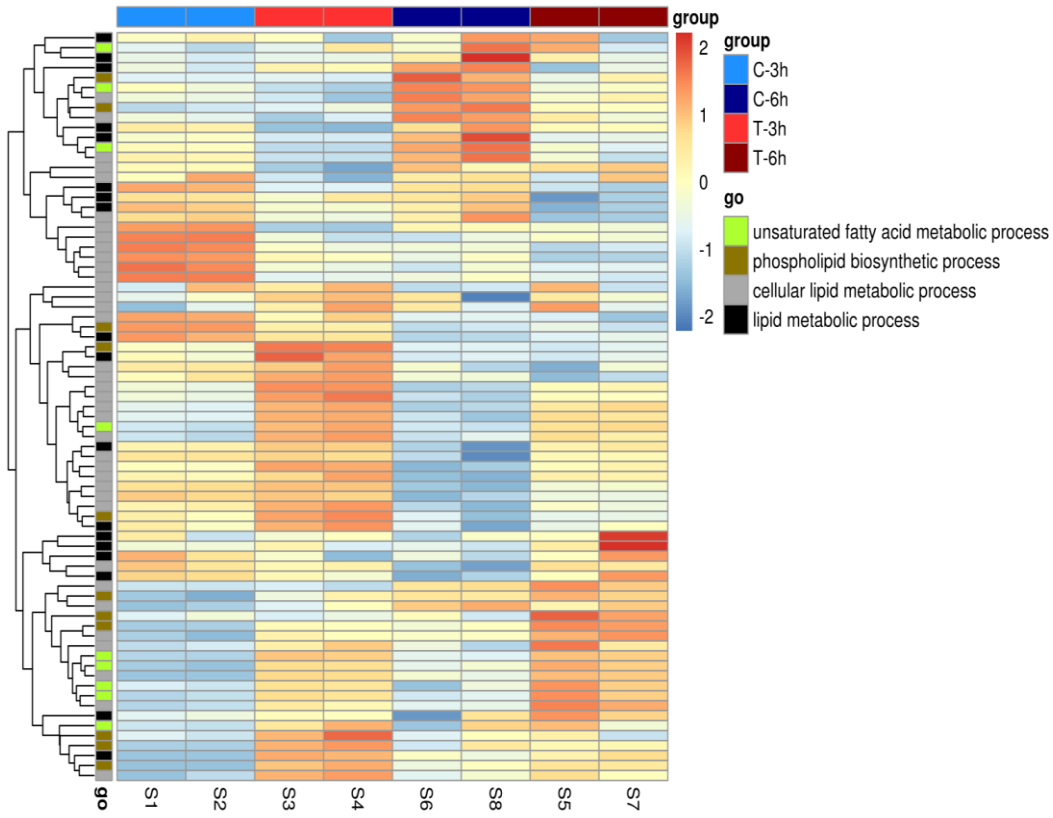
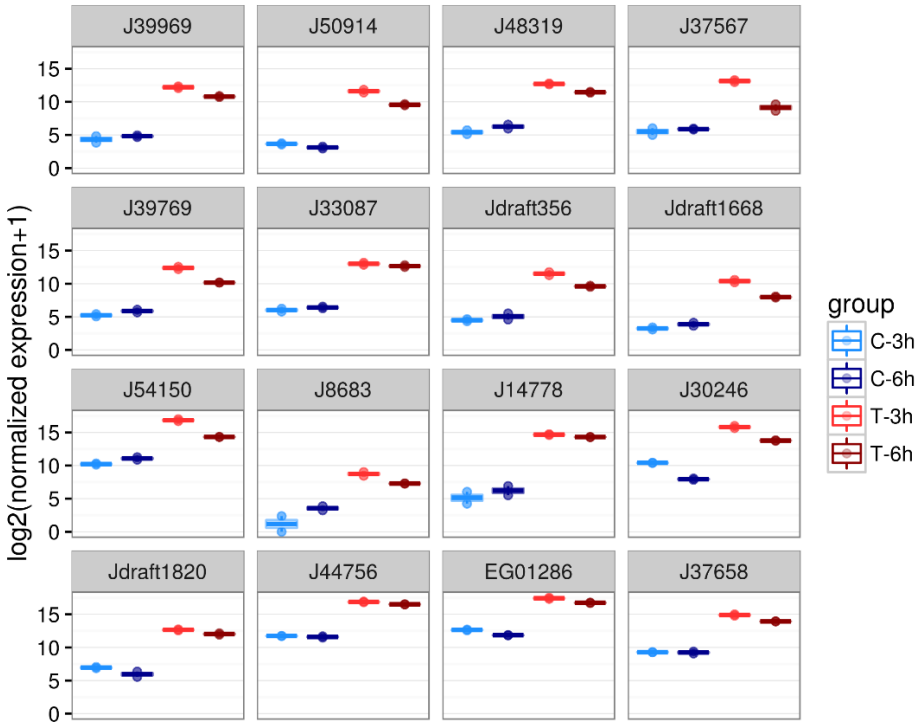
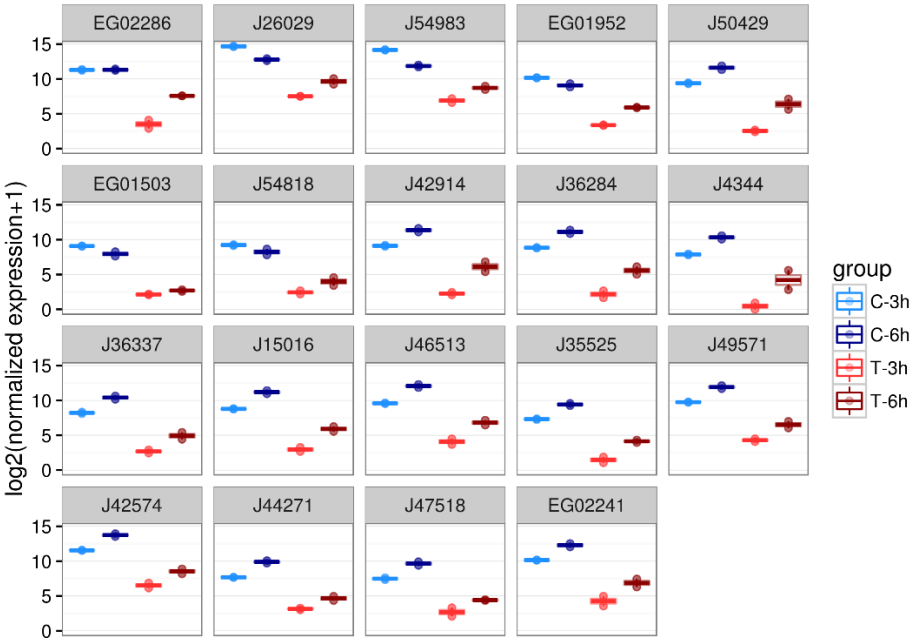


Figure 3.5: Heatmap of genes associated with lipid metabolic processes GO group in DMSO control (C) and DD treated (T) at 3 hr and 6 hr. (S1 and S2 -- DMSO control replicates at 3 hr; S3 and S4—DD treated replicates at 3 hr; S6 and S8—DMSO control replicates at 6 hr; S5 and S7—DD treated replicates at 6 hr)



JGI transcript ID	JGI Annotation
transcript:Phatr3_J39969.t1	No annotation
transcript:Phatr3_J50914.t1	NADH:flavin oxidoreductase/NADH oxidase
transcript:Phatr3_J48319.t1	No annotation- possibly involved in Cell cycle control
transcript:Phatr3_J37567.t1	No annotation
transcript:Phatr3_J39769.t1	No annotation
transcript:Phatr3_J33087.t1	No annotation
transcript:Phatr3_Jdraft356.t1	No annotation
transcript:Phatr3_Jdraft1668.t1	No annotation
transcript:Phatr3_J54150.t1	heat shock protein Hsp20
transcript:Phatr3_J8683.t1	Peptide methionine sulfoxide reductase
transcript:Phatr3_J14778.t1	ABC transporter like/ ATP-GTP binding site/AAA+ATPase core
transcript:Phatr3_J30246.t1	Zinc-containing alcohol dehydrogenase superfamily
transcript:Phatr3_Jdraft1820.t1	No annotation
transcript:Phatr3_J44756.t1	ATP/GTP-binding site motif A (P-loop)/ ABC transporter like
transcript:Phatr3_EG01286.t1	No annotation
transcript:Phatr3_J37658.t1	Glutathione S transferase

Figure 3.6: Top upregulated genes at 3 hr and 6 hr in response to DD treatment (T) compared to DMSO solvent control (C). (padj or q <0.05)



JGI transcript ID	JGI Annotation
transcript:Phatr3_EG02286.t1	No Annotation
transcript:Phatr3_J26029.t1	Nitrate transporter
transcript:Phatr3_J54983.t1	possible - Nitrate reductase [NADH] (NR)
transcript:Phatr3_EG01952.t1	No Annotation
transcript:Phatr3_J50429.t1	Glycosyl transferase, family 8
transcript:Phatr3_EG01503.t1	No annotation
transcript:Phatr3_J54818.t1	putative ferric reductase
transcript:Phatr3_J42914.t1	S layer protein (SLH domain)/von Willebrand factor and related coagulation proteins
transcript:Phatr3_J36284.t1	Chromosome condensation complex Condensin, subunit H/ Barren
transcript:Phatr3_J4344.t1	FOG: Leucine rich repeat
transcript:Phatr3_J36337.t1	Myb, DNA-binding
transcript:Phatr3_J15016.t1	Myb, DNA-binding
transcript:Phatr3_J46513.t1	hypothetical protein
transcript:Phatr3_J35525.t1	No annotation
transcript:Phatr3_J49571.t1	Leucine-rich repeat/ on Willebrand factor
transcript:Phatr3_J42574.t1	Carbonic anhydrase, eukaryotic
transcript:Phatr3_J44271.t1	Nuclear protein SET (chromosomal proteins modulating gene activities and/or chromatin structure)
transcript:Phatr3_J47518.t1	hypothetical protein
transcript:Phatr3_EG02241.t1	No annotation

Figure 3.7: Top downregulated genes at 3 hr and 6 hr in response to DD treatment (T) compared to DMSO solvent control (C). (padj or q <0.05)

Chapter 4: Conclusions and Future Works

The overall goals of this study were to use various analytical, biochemical and molecular techniques to understand lipid changes and the regulation of these changes at the transcriptome level in the diatom *Phaeodactylum tricornutum* in response to the model elicitor, decadienal (DD), which is induced in defense responses. Diatoms are the most abundant and unique photosynthetic phytoplankton which are known for their great flexibility to adapt to rapidly changing environmental conditions. In the recent years there have been number of studies documenting the roles of nutrient stress such as nitrogen and phosphorous, various heavy metals, temperature, pH etc. on remodeling of lipids in the diatoms and coordination of various metabolic pathways at transcriptome and proteome levels regulating these lipid changes. However limited studies have been done in the area of biotic stress such as cell damage or wounding in diatoms. Diatoms respond to cell damage by releasing bioactive oxylipins (oxygenated lipids) from membrane phospholipids, which have varied effects on diatoms themselves as well as on grazers. A key goal of this study was to understand how healthy/undamaged diatoms would respond (in relation to overall lipid and transcriptome changes) to these oxylipins released from the site of herbivory that might play a role in their defense, and that would likely be important in determining phytoplankton community structure, especially during the time of algal blooms. Since most diatoms are important source of lipids and unsaturated fatty acids, this study may inform a better use of their biomass for making cost effective commercial products.

Our data on determining lipid changes in diatom *P. tricornutum* in response to external application of sub lethal dose of DD in a short time scale (3 hr-6 hr) showed that

all the fatty acids were downregulated at 3 hr except 18:1, which remained unchanged, whereas at 6 hr there were increases in 18:1 and 18:2 fatty acids but a decline in other fatty acids. DD did not have an effect on abundant chloroplast lipids (MGDG, DGDG and SQDG) whereas it did have an effect on extra plastidic membrane phospholipids, among which increases in PC, PE and decline in PS, PG and LPG levels were seen. Sterols declined initially at 3 hr but remained unchanged at 6 hr. Analysis of molecular ion species of PC, PE, Lyso PC and Lyso PE membrane lipids showed that there was a shift towards saturated and monounsaturated fatty acids compared to polyunsaturated fatty acids. It was suspected that these changes in membrane lipids might alter membrane properties, so membrane permeability of three cellular dyes with varied structures and molecular masses were evaluated. Two out of three dyes had reduced uptake into the cells indicating that both structure of dye and altered membrane lipids possibly determines the permeability of molecules into the cells.

Further studies are needed to understand changes in other membrane properties that might help cells survive the DD stress. It would be interesting to check permeability of DD or other oxylipins using tagged fluorescent probes or by techniques of derivatization and mass spec analysis to determine if changes in membrane lipids in these cells alter permeability of various oxylipins into the cells. It would help cells survive further toxic doses of these compounds after an initial sublethal dose as a defense mechanism to attack by grazers or during the times of algal blooms and hence help determine plankton community structure. Further incubation experiments with DD induced *P. tricornutum cells* (with changed lipids) with grazers would provide more knowledge on relation of food quality to that of feeding preference of grazers.

Another area of future study would be to determine if DD induced changes in membrane lipids might lead to downstream production of any compounds that might act as a relay to signal distant healthy cells during time of grazer attack. Since *P. tricornutum* is known to produce non-volatile compounds (9-ONDE and 12-ODTE) during mechanical cell damage and these compounds have been shown to have similar effects on grazers as that of aldehydes such as DD, it would be interesting to check if DD stress can induce release of these or any other oxylipins downstream as a defense response.

Another future study would be to understand the role of increased Ca^{+2} levels and ROS/NO production in DD induced lipid changes that would further strengthen knowledge of defense mechanisms in these organisms due to environmental stresses.

Transcriptome studies in *P. tricornutum* in response to sub-lethal decadienal dose in short time scale (3 hr-6 hr) showed that various metabolic processes were affected indicating coordinated response of various pathways to adapt to DD stress. Genes in processes such as photosynthesis, ion transport, DNA metabolism and chromatin/chromosome organization were downregulated, whereas genes in processes such as RNA metabolism, carbohydrate and protein metabolism, lipid metabolism, translation and amino acid and protein synthesis were upregulated. The regulation of various processes indicated allocation of carbon and other resources to required processes necessary for survival against decadienal stress. Genes involved in initiating fatty acid synthesis were downregulated, which correlated to observed decline of fatty acids levels, but a number of genes involved in unsaturated fatty acid synthesis in cytosol and ER were upregulated, indicating that observed increases in 18:1 and 18:2 fatty acid levels might be regulated at transcriptome level. Genes involved in TAG synthesis pathway along with lipid degradation and oxidation genes were downregulated supporting observed minimal

increases in TAGs and more prominent increases in PC and PE membrane phospholipids. There might be a regulation at translational levels showing a need of analysis of proteome of *P. tricornutum* under similar conditions. Among the top category of upregulated genes were those involved in ROS metabolism, stress and antioxidant defense mechanisms, which correlates well to studies done on known ROS production in *P. tricornutum* cells in response to decadienal. Further we plan to choose 3-5 genes mostly related to lipid metabolism pathway to analyze gene expression via independent qPCR experiments to confirm RNA Seq expression data. Further studies on proteome under similar conditions would help integrate transcriptomics and proteomics data to get better understanding of regulation of metabolic pathways to adapt to DD stress. In addition, the mechanism of how PUAS communicate the signal via second messengers and effectors need to be evaluated further. Our study provides insights on DD induced changes in lipids composition in the cells and role of lipid metabolism pathway genes in regulating lipid changes at transcriptome level within a short time scale. Elucidation of biochemical and molecular responses of phytoplankton to oxylipin stress can improve our understanding of mechanisms of defense in these organism and overall structure of phytoplankton community during algal blooms. It also informs about intelligent use of phytoplankton biomass for making cost effective commercial products.

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